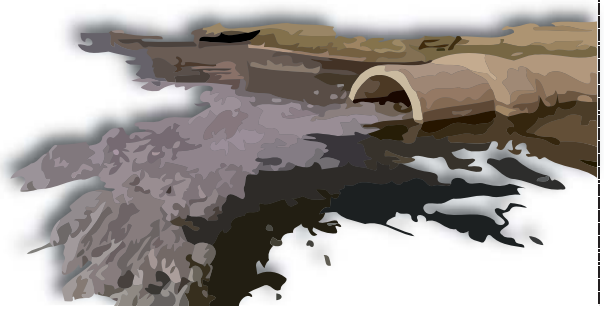
JACUMAR
JUNTA NACIONAL ASESORA DE CULTIVOS MARINOS

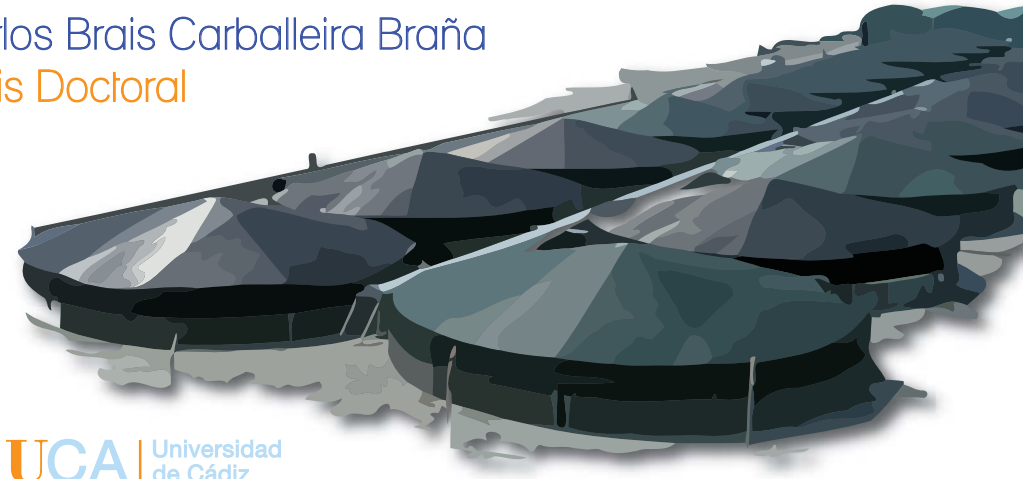


Bases científicas para el diseño de un plan de vigilancia ambiental para las piscifactorías marinas instaladas en tierra | Tesis Doctoral | Carlos Brais Carballeira Braña | UCA

Bases científicas para el diseño de un plan de vigilancia ambiental para las piscifactorías marinas instaladas en tierra

Carlos Brais Carballeira Braña

Tesis Doctoral



UCA | Universidad de Cádiz

UNIVERSIDAD DE CÁDIZ

FACULTAD DE CIENCIAS DEL MAR Y AMBIENTALES

BASES CIENTÍFICAS PARA EL DISEÑO DE UN PLAN DE VIGILANCIA AMBIENTAL PARA PISCIFACTORÍAS MARINAS INSTALADAS EN TIERRA

Memoria para optar al título de Doctor

presentada por

Carlos Brais Carballeira Braña

Cádiz, 2012

Esta Tesis Doctoral ha sido realizada dentro del Grupo de Investigación Contaminación de Sistemas Acuáticos (C.O.S.A., RNM375), perteneciente al Plan Andaluz en colaboración con el Grupo de Ecotoxicología de la Universidad de Santiago de Compostela.

La labor de dicho grupo se desarrolla en la Facultad de Ciencias del Mar y Ambientales de la Universidad de Cádiz, en el marco de las actividades de investigación de la Cátedra Santander-UNESCO UNITWIN/WiCop, en su área de calidad ambiental.

El trabajo que se resume en esta memoria ha sido financiado principalmente por el Ministerio de Agricultura y Pesca mediante del proyecto *Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos de métodos y medidas para estudios ambientales en acuicultura marina* (INDAQUA) (PNCM/IND/08), concedido a través de la Junta Nacional Asesora de Cultivos Marinos (JACUMAR) dentro del Plan Nacional de cultivos Marinos. En el desarrollo del proyecto INDAQUA intervinieron seis CCAA, aunque la temática desarrollada en esta tesis se centra exclusivamente en el estudio de las piscifactorías marinas instaladas en tierra en Galicia.

D. **T. ÁNGEL DEL VALLS CASILLAS**, Catedrático de la Universidad de Cádiz y Dña. **M. LAURA MARTÍN DÍAZ**, Profesora Titular del Departamento de Química Física de la Universidad de Cádiz, como sus directores,

HACEN CONSTAR

Que la presente memoria, titulada **Bases científicas para el diseño de un plan de vigilancia ambiental para piscifactorías marinas instaladas en tierra**, presentada por D. **Carlos B. Carballeira Braña** resume su trabajo de Tesis Doctoral y reúne los requisitos legales y las condiciones de originalidad y rigor científico, por lo que autorizan su presentación y defensa para optar al grado de Doctor en Ciencias del Mar por la Universidad de Cádiz.

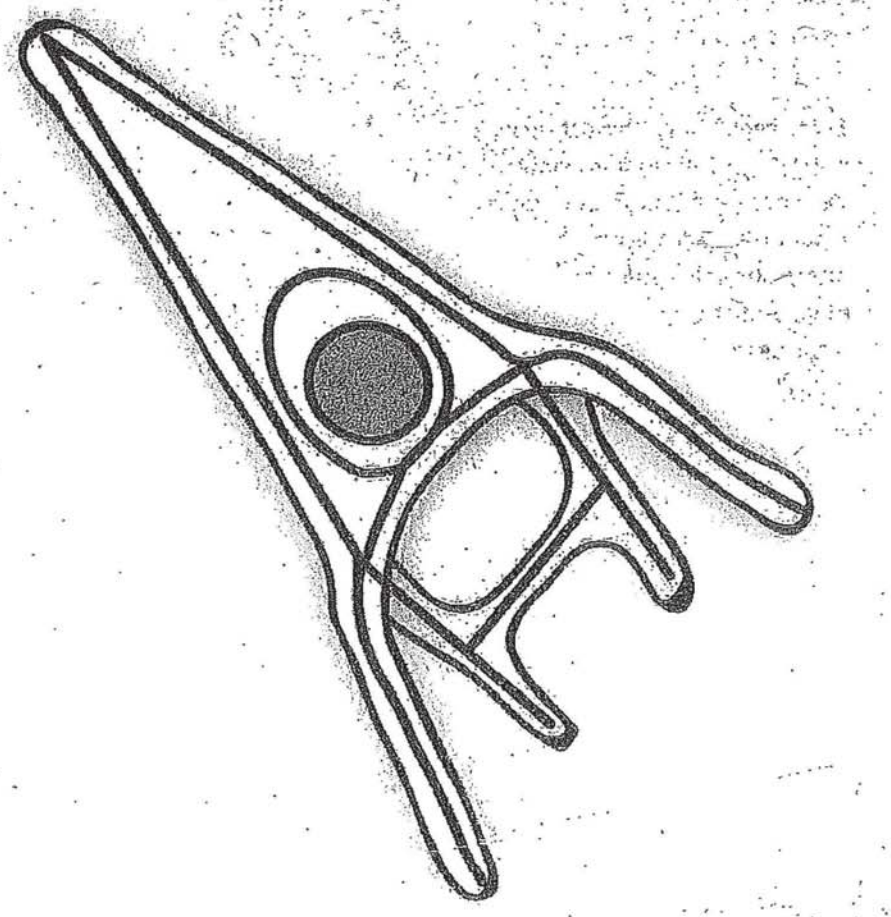
Cádiz, 15 de Noviembre de 2012

Fdo.: T. Ángel del Valls Casillas Fdo.: M. Laura Martín Díaz

LA FIRMA INVITADA

Carnaval con espinas

Carlos Brais Carballeira Braña
Departamento de Química Física. Facultad de Ciencias del Mar y Ambientales (UCA)



La erizada anuncia con ritmos tamborileros y sabores marinos el comienzo del Carnaval, programado este año para finales de enero. Es en estas fechas cuando este curioso invertebrado muestra todo el esplendor de sus gónadas, cuyo fin de desarrollo viene determinado por un ligero incremento de la temperatura del agua.

El erizo de mar ha supuesto un aporte energético como alimento desde el Neolítico, y debido a su amplia extensión, su uso gastronómico no es único de Cádiz, siendo Japón el mayor productor y consumidor. Aunque dudo mucho que lo disfruten con tanta gracia y salero como en el Barrio de la Viña.

Este internacional equinodermo (del latín piel con espinas), fuera del plato, pasa desapercibido a los ojos de la sociedad. Por ejemplo, jamás un niño jugará a ser un erizo, en lugar de eso, los pequeños prefieren interpretar el papel de los grandes depredadores y subconscientemente se colocan en la posición más alta de la cadena alimentaria. Para los erizos de mar la situación es bastante más complicada que para los leones. En primer lugar, estos animales son un manjar para muchas otras especies. Es curioso que un pez como el pez ballesta se pueda alimentar de un erizo de mar y que a nosotros aun siendo Homo Sapiens nos cueste cogerlo con la mano. No quiero ni imaginarme cogerlo con la boca y luego morderlo a pinrel. Se me ocurren también las nutrias marinas, que los cogen con su mano palmeada y los revientan contra las rocas como si de nueces se tratasen. Pero sin duda lo que realmente amenaza al erizo es la contaminación del agua (son altamente sensibles porque el agua de mar que lo rodea también rodea sus órganos interiores, que quedan expuestos a cualquier contaminante que haya aumentado su concentración hasta niveles dañinos), la destrucción o explotación de las algas, que son su principal alimento, la introducción de especies exóticas y la sobreexplotación, incluyendo la pesca furtiva.

El erizo tiene que vivir cinco años para convertirse en adulto y reproducirse por primera vez. Es triste compararlo con un gato, que como se te escape con más de un año de vida puede convertirte en abuelo de hasta diez gatitos. Para que una población de erizos se mantenga es necesario que se respeten al menos la mitad de los ejemplares, ya que las crías sólo consiguen adherirse a la roca (fundamental para su supervivencia) si previamente la han limpiado los adultos. No estoy diciendo con esto que tenemos que dejar de comerlo o de celebrarlo, porque, aunque no me gusten especialmente sus gónadas, como buen gallego (pero censado en Cádiz) mi pasión por los mariscos me lleva todos los sábados al nuevo Mercado de Abastos del casco antiguo, y si hay marisco vivo, no lo dudo y me doy un homenaje.

Muchos lo desconocen, pero el erizo de mar ha hecho grandes aportaciones al desarrollo del ser humano, quien no parece agradecer dichos acontecimientos. Fue en el 50º aniversario del descubrimiento de la doble hélice del ADN por James Watson y Francis Crick cuando se mencionó que fue ADN extraído del esperma del erizo el primero que vieron estos científicos. En Francia, ya en 1930 se fabricaba a partir de sus gónadas una conserva que llamaban caviar de "oricios" y que consideraban comparable al caviar mundialmente codiciado fabricado con huevas de esturión.

Hay veces que me acerco a la Punta de San Felipe y al ver esos peinados afilados y engominados hacia todas direcciones me acuerdo de mi querido erizo. Y esto tiene su explicación. Esta especie (Paracentrotus lividus o erizo de mar común) comparte un antepasado común con el ser humano. Ese ancestro vivió hace 540 millones de hace años y dio lugar a los deuteróstomos, un superfílum de animales donde encontramos ambas especies.

Y acabo finalmente por explicar en qué medida el erizo me ayuda a mí, como científico ecólogo. Me encuentro en la fase final de mi doctorado. A lo largo de los últimos tres años he usado el erizo de mar como indicador de la contaminación. La técnica que empleo consiste en crear embriones o larvas a partir de los gametos de dos individuos adultos (un macho y una hembra). Una vez formadas las larvas, las expongo a distintas disoluciones con productos contaminantes o vertidos y pasados dos días bloqueo el desarrollo de las larvas, que en esos momentos y si todo está en orden tendrán forma de nave espacial (ver dibujo). Posteriormente, observo al microscopio las deformidades que puedan tener las larvas y expreso el resultado como el porcentaje de larvas deformes que existen en dicha muestra. Y con ese sencillo proceso podré saber si el agua de playa Victoria está más o menos limpia que la de La Caleta.

Citas

Célebre
Los imposibles de hoy serán posibles mañana.
Konstantin Tsiolkovski

De actualidad
La pregunta es importante sólo si la respuesta es correcta
Karate kid

Personal
La dilución no es la solución
Carlos Carballeira

Agradecimientos

Cai

En primer lugar agradecer la ayuda y el apoyo proporcionado por mi director, el catedrático Ángel Del Valls Casillas, y mi codirectora, la Dra. Laura Martín Díaz no sólo durante la coordinación de esta tesis sino también a lo largo del desarrollo del Máster Erasmus Mundus in Water and Coastal Management. Siempre me han animado cuando un experimento resultara fallido. Gracias por vuestro apoyo, dedicación y confianza.

Me gratifica saber que en el departamento de Química-física me encuentro dulce y abundantemente rodeado de compañeras maravillosas: Luciane, Araceli, Loli, Gabriela, Rocío, Judith,… Tampoco quiero olvidarme de la gente que estuvo en el departamento antes que yo y que se fueron al poco de conocernos; Natalia, Nuria, Carmen, Mamen,…

Quique, Claudio ¡¡¡sois unos cracks, no cambiéis!!!

Carmen López, compañera y amiga por su incansable esfuerzo para ayudar a todos los que la rodeamos. A la Dra. María José, porque siempre pude contar con ella para cualquier cosa, y por ser ella la que me introdujo al conocimiento de los erizos de mar y sus aplicaciones y a Mario, por ser tan buena persona.

A todos aquellos que fueron mis compañeros de piso por una convivencia agradable y tranquila y a los compañeros del máster Erasmus Mundus de los últimos 5 cursos por los buenos momentos compartidos y por tratarme como uno más del grupo.

Manoela, los dos sabemos que somos de la misma familia.

Muchísimas gracias a aquellos que habitan en la puerta de enfrente; Juanma, Cori, Marina, Rosa, Ivy y Pablo, por haber compartido material, consejo y unas risas. Parte de esta tesis no se podría haber hecho sin vosotros.

Julia, no sólo incansable en la tarea de soportarme sino que también reúnes todos los puntos resaltados anteriormente; somos de la misma familia, eres una crack y, por supuesto, también formas parte de esta tesis. Gracias.

A los brasileños; Daniel, Lia, Sadao,… porque ellos ponen la samba y las caipirinhas. No sé quién dijo que en la vida de un gallego siempre tiene que haber un brasileño, pero tenía razón, nos complementamos.

Aquellos que no pertenecen al mundo de la ciencia pero que también han seguido la trayectoria de esta tesis, porque son mis amigos y con ellos comparto gran parte del tiempo que tengo libre. Gracias Gabi por la espiritualidad y por el Palmar. Javi por las películas, los comics y el saber estar. Chipirón por ser incansable, animado y por los homenajes. Toti por ser el anfitrión perfecto. Javi y Ana, por poder contar con vosotros.

A todos los Erasmus que pasaron por el laboratorio haciendo prácticas y proyectos de Master; Nick, Saq, Robbie, Alex,… por ser el resquicio que me quedaba de la vida de estudiante y por su ayuda en el laboratorio. Lo siento y gracias por haberme ayudado, entre otras cosas, a etiquetar 50.000 eppendorffs, 5.000 viales, 4.000 tubos de ensayo y 2.000 bolsas, es duro.

Miña Terra Galega

A Mi madre, Maribel, porque hacemos un buen equipo, porque sabe de qué va todo esto de la universidad, por su iniciativa, y aunque ya haya pasado bastante tiempo desde el último, gracias por los paquetes de comida. Gracias a MRW porque sin ti no hubiesen sido gratis. No podría olvidarme de mi padre, Alejo Carballeira Ocaña, por ser el pilar central de esta tesis y por ser un ejemplo a seguir tanto en la vida personal como en la comunidad científica.

Un gracias especial para mis padres por haberme guiado en cada decisión importante de mi vida.

A Susana, mi segunda madre. Por estar ahí toda mi vida y por las charlas durante las comidas de las 2 del mediodía.

Quiero también agradecer al equipo de Ecotoxicología de la Universidad de Santiago, porque sin vosotros esta tesis no se hubiese realizado, porque fundasteis los cimientos del investigador que soy y porque me habéis enseñado como funciona un laboratorio. Un especial agradecimiento a Jesús, Ángel, Ana, Merche, Cris, Inés,… por vuestra preocupación y dedicación. Es un auténtico privilegio haber podido trabajar a vuestro lado.

Adri y Romi mis queridos compañeros gallegos de Cádiz, está claro que compartimos patria.

A la pandilla de Santiago; Chuky, Álvaro, Manu, Eijo, Nimo, Stefie, Nagüer, Toñi, Patri, Joaco, Miki, Brais, Fanny, Cris, Gloria,… No os preocupéis, celebraremos la tesis también en Santiago.

A toda mi familia, estoy más que orgulloso de mis apellidos.

A aquellos marineros (Los Pepes, Fran y Fran junior) que me han ayudado a realizar los muestreos en barco y que dirigieron el barco a buen puerto incluso los días de fuerte marejada.

A Joaquín Espinosa por haberme dejado su "Ferrari" y por los conocimientos transmitidos sobre histopatología.

Gracias a la UTIA de la Universidad de la Coruña porque vuestros análisis de N^{15} fueron imprescindibles para mejorar la calidad de la tesis. Gracias a

Celina por ser tan agradable y por dejarme usar su báscula de precisión. Gracias también al Dr. Fabregas por las algas y el medio de cultivo.

Organizaciones

Al Ministerio de Educación, la Xunta de Galicia, la Junta de Andalucía y la Universidad de Cádiz por las ayudas y becas proporcionadas, fueron, junto a los paquetes de comida de mi madre, mi sustento durante la realización de esta tesis.

A la junta nacional de cultivos marinos (JACUMAR) por financiar el proyecto INDAQUA, "*Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos y medidas para estudios ambientales en acuicultura marina*" *y a la Dirección Xeral de Innovación e Desenvolvemento Pesqueiro de la Consellería de Pesca e Asuntos Marítimos de la Xunta de Galicia por haber contado con nuestra participación en el desarrollo del proyecto.*

Gracias a los creadores del Adobe Illustrator, SPSS, Excel, Endnote, R,… porque sin ellos este trabajo hubiera llevado muchos más años y porque gracias a ellos me manejaré mejor en futuros trabajos.

Acrónimos y abreviaturas

δ	Escala delta
$\delta^{15}N$	Relación o señal isotópica de nitrógeno
AhR	Aryl hydrocarbon receptor
AIC	Akaike´s information criterion
Al	Aluminio
AMOX	Amoxicilina
AMP	Ampicillina
BEQI	Benthic Ecosystem Quality Index
CACYTMAR	Centro Andaluz de Ciencia y Tecnología Marina
Cd	Cadmio
CETESB	Companhia de Tecnologia de Saneamento Ambiental
CETGA	Centro Tecnológico de Acuicultura de Galicia
Chla	Clorofila a
Chlb	Clorofila b
Cr	Cromo
CSTT	Commision for Scientific Technical Terminology
Cu	Cobre
CYP	Cytochrome P450
DEPOMOD	Depositional Modelling (relativo a piscifactorias marinas en jaulas)
DBF	Dibenzylfluorescein dealkylase
DNA	Deoxyribonucleic acid
DO	Dissolved oxigen
EC	Effective concentration
e.g.	Exempli gratia – Por ejemplo
ELD	Embryo larval development
EM	Estación de muestreo
EMP	Environmental Monitoring Plan
EQO	Environmental Quality Objective
ERA	Environmental Risk Assessment
EROD	Ethoxyresorufin-O-deethylase
ESBG	Environmental Specimen Bank of Galicia
FAO	Food and Agriculture Organization
FLU	Flumequina
GESAMP	Group of Experts on the Scientific Aspects of Marine Environmental Protection

GPX	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferase
Hg	Mercurio
HNO_3	Ácido nítrico
HPLC	High Performance Liquid Chromatography
I	Input
ICES	International Council of the Exploration of the Sea
i.e.	Id est – Es decir
INH	Inhibition index
INDAQUA	Selección de Indicadores, Determinación de Valores de Referencia, Diseño de Programas y Protocolos de Métodos y Medidas Para Estudios Ambientales en Acuicultura Marina
JACUMAR	Junta Asesora de Cultivos Marinos
KCl	Cloruro potásico
KNO_3	Nitrato potásico
LBMFF	Land-based marine fish farm
LOE	Line of evidence
LOEC	Lowest observed effect concentration (significativa frente al control)
LPO	Lipid peroxidation
MF	Malformed/Malformations
MS/MS	Espectrometría de masas en tándem
n	Número de replicados
N	Nitrógeno
N_2	Nitrógeno molecular
NaCl	Cloruro sódico
NaClO	Hipoclorito sódico
NIOO	Nederlands Instituut voor Oecologish Onderzoek
NH_3	Amoniaco
NH_4^+	Ión amonio
NH_4Cl	Cloruro de amonio
$(NH4)_2SO_4$	Sulfato de amonio
Ni	Níquel
NO_2^A	Ión nitrito
NO_3^A	Ión nitrato
NOEC	No observed effect concentration (significativa frente al control)
NW	North West

O	Output
O_2	Oxígeno molecular
OECD	Organisation For Economic Co-operation and Development
OSPAR	Oslo and Paris Convention
OTC	Oxitetraciclina
p	Significación estadística
PAH	Polycyclic aromatic hydrocarbon
Pb	Plomo
PCB	Polychlorinated biphenyls
pCO_2	Presión parcial de CO_2
PEEP	Potential Ecotoxic Effects Probe
POM	Particulate organic matter
PVA	Plan de Vigilancia Ambiental
R	Coeficente de ajuste de la recta de regresión
ROS	Reactive oxygen species
SEPA	Scottish Environmental Protection Agency
SFD	Sulfadiazina
SMA	Standard major axis
SQT	Sediment Quality Triad
SS	Suspended solids
SS	Sampling site
ST	Streptomycin
TOC	Total organic carbon
TU	Toxic unit
UCA	Universidad de Cádiz
UD	Undeveloped
UNESCO	United Nations Educational, Scientific and Cultural Organization
UNITWIN	University Twinning and Networking
USC	Universidad de Santiago de Compostela
USEPA	United States Environmental Protection Agency
UTIA	Unidad de Técnicas Instrumentales de Análisis
UV	Ultraviolet
WFD	Water Framework Directive
WICOP	Wise Coastal Practices for Sustainable Human Development
WID	Weighted Index of Damage
WOE	Weight of evidence
Zn	Zinc
$ZnSO_4$	Sulfato de zinc

Índice de contenidos

LA IMPORTANCIA DE UN PLAN DE VIGILANCIA AMBIENTAL PARA LA PISCICULTURA MARINA INSTALADA EN TIERRA

1.1. Situación general de la Acuicultura

La acuicultura aprovecha el espacio y los recursos acuáticos para el cultivo de especies, básicamente peces, moluscos, crustáceos, algas y plantas acuáticas, con fines económicos. Esta actividad surge como respuesta a la elevada presión ejercida sobre los recursos del medio (e.g. sobreexplotación pesquera) y a la creciente demanda de alimento por parte de la población, necesitando la intervención del hombre para incrementar la producción acuícola mediante la concentración, alimentación y protección de dichas poblaciones (FAO, 2012).

En la actualidad la acuicultura es el sector de producción de alimentos con el crecimiento más acelerado y representa casi el 50% de los productos pesqueros mundiales destinados a la alimentación (FAO, 2012). Este rápido crecimiento requiere de un conocimiento proporcional sobre todos los temas relacionados tanto con el cultivo como con sus efectos ambientales, con el fin de realizar una gestión responsable de esta actividad. Constantemente se crían nuevas especies y emergen nuevos productos químicos y alimentarios (piensos), además, los tipos y las cantidades de éstos varían según el país (Costello et al., 2001).

La acuicultura se ha presentado en los medios como una industria no contaminante, sin embargo, como ocurre en el caso de otras industrias de producción animal intensiva, esta actividad es potencialmente contaminante debido al elevado volumen de productos residuales que se generan y que a menudo terminan en ríos, estuarios y mares, con posibles efectos tóxicos y tróficos que perjudican a los ecosistemas, procesos y organismos acuáticos. Entre estos efectos se encuentran la alteración de las características físicas, químicas y biológicas de la columna de agua y de los

fondos marinos, la atracción de especies oportunistas o la disminución de la biodiversidad.

No obstante, la acuicultura ha de ser respetuosa con el medio ambiente para garantizar su sostenibilidad porque necesita de una buena calidad del medio del cual obtiene los recursos naturales (Roque D'Orbcastel et al., 2004). Para integrar la acuicultura dentro del ecosistema es necesario tener en cuenta todas las interacciones que ésta tiene con el medio ambiente (Pillay and Kutty, 2005; Read and Fernandes, 2003).

Aunque existen muchas iniciativas, e.g. guías metodológicas y bases de datos físico-químicos y toxicológicos para la vigilancia ambiental creadas por agencias medioambientales (Roque D'Orbcastel et al., 2004; Lazard et al., 2011), la ausencia de un enfoque ecotoxicológico a la hora de estudiar las repercusiones de esta actividad en el medio y la poca transparencia del sector acuícola dificultan el correcto control del impacto ambiental generado por la acuicultura (Sapkota et al., 2008; Burridge et al., 2010; Crane et al., 2007; FAO, 2010).

1.2. La acuicultura en España

La acuicultura en España está demostrando ser una actividad en expansión. Según el Ministerio de Medio Ambiente, Medio Rural y Marino y la FAO, España es el país con mayor producción acuícola de la Unión Europea, principalmente debido al cultivo vertical de bivalvos. Sin embargo, la producción acuícola española total no ha crecido en la última década debido a que el 75% de ésta corresponde al cultivo de mejillón, cuya producción anual depende principalmente de las mareas rojas que afectan a las rías gallegas. Por el contrario, el creciente interés por parte de inversores, empresas y administraciones españolas y de otros países por la piscicultura española permitió que el cultivo de peces marinos doblase su producción durante los años 2000-2008 (JACUMAR, 2012).

En la actualidad existe una notable disparidad de criterios en cuanto a los contenidos, diseño y ejecución de los Planes de vigilancia ambiental (PVA) de los cultivos marinos en las distintas CCAA que componen el territorio español. Esta heterogeneidad lleva ligada además una considerable

carencia de base científica para la elaboración de los PVA, y en la mayoría de los casos no se sigue una estrategia adecuada de gestión ambiental. Esto supone, en muchos casos, la generación de información poco útil tanto para la empresa como para la administración. Para contribuir a la solución de estas deficiencias, en base a los resultados obtenidos en el proyecto JACUMAR[1], recientemente el Ministerio de Agricultura, Alimentación y Medio Ambiente ha presentado una "Propuesta metodológica para la realización de los planes de vigilancia ambiental de los cultivos marinos en

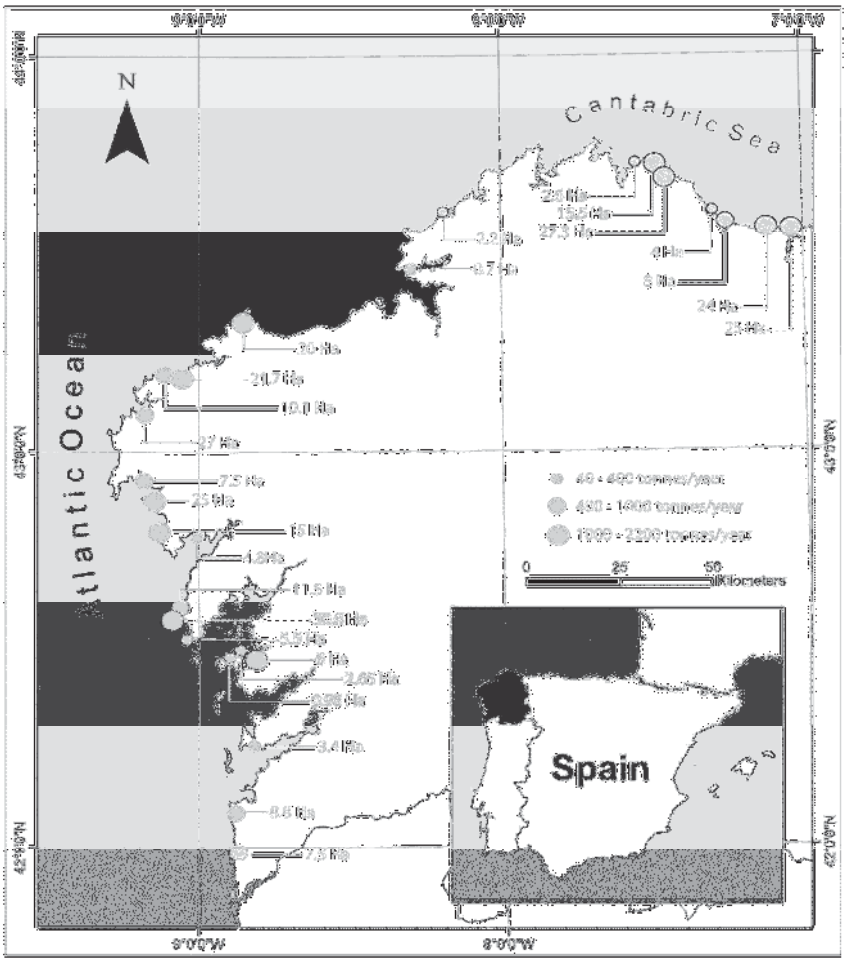


Figura 1. Localización, superficie y producción de las piscifactorías marinas intensivas instaladas en tierra del noroeste peninsular (Galicia).

jaulas flotantes"(JACUMAR, 2012). Lamentablemente, esta propuesta

[1]El proyecto financiado por la Junta de Cultivos Marinos (JACUMAR) titulado "Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos de métodos y medidas para estudios ambientales en acuicultura marina", desarrollado entre 2008-2011, con la participación de centros de investigación de 6 Comunidades Autónomas y 20 empresas del sector, se ha dirigido a establecer las bases sobre las que diseñar protocolos y planes de seguimiento ambiental de la acuicultura, con el propósito de facilitar a las empresas el desarrollo de los estudios ambientales pertinentes, y simplificar a las administraciones la gestión ambiental relativa a la acuicultura marina.

metodológica no es aplicable a las instalaciones de acuicultura marina intensiva en tierra por sus especiales características y de cuyo impacto ambiental existe una amplia laguna de conocimientos respecto a la acuicultura en jaulas.

Desde sus comienzos en la década de los 80, la producción española de rodaballo (*Psetta maxima* Linnaeus, 1758), principalmente desarrollada en el noroeste peninsular ha crecido de forma exponencial, sobre todo en los últimos años. La producción global de peces planos se incrementó significativamente de 26.300 toneladas en 2000 a 148.800 toneladas en 2008, con China y España como los principales productores. La particularidad del cultivo del rodaballo es su producción intensiva en granjas marinas instaladas en tierra (LBMFF, Land Based Marine Fish Farm) localizadas por toda la costa gallega. En la Figura 1 se muestra un mapa de Galicia con la localización, producción y superficie de las piscifactorías destinadas al cultivo de rodaballo y ocasionalmente de lenguado (*Solea solea* Linnaeus, 1758).

1.3. Características del cultivo intensivo en tierra

Todas las fases del cultivo marino en tierra se realizan en instalaciones terrestres (incluyendo el engorde) que gracias al uso de sistemas de aireación para mantener el agua saturada de oxígeno, comederos automáticos, estufas y otros dispositivos permiten tener un mayor control de las condiciones en las que viven los peces.

La elevada densidad media de los peces dentro de los tanques de cultivo facilita la transmisión de enfermedades. Para evitar dicha propagación es necesario mantener unas condiciones de higiene estrictas que normalmente se consiguen mediante una buena desinfección de las instalaciones y/o mediante el uso preventivo de medicinas (Figura 2).

En la mayor parte de los casos el agua de mar que llena dichas piscinas se bombea en circuito abierto. De esta manera, el medio de cultivo se encuentra en constante renovación, lo que genera unos elevados costes de bombeo y por tanto, la necesidad de instalar las granjas pegadas a la costa. Por este motivo los emisarios de entrada y salida del agua están

relativamente cerca uno del otro por lo que, en principio, podrían generarse retroalimentaciones negativas. Para evitar que el agua de entrada se contamine con el agua de salida se pueden construir los emisarios alejados y a distintas profundidades, instalar sistemas de circuito cerrado o se pueden ubicar las instalaciones en costas muy expuestas. Las granjas terrestres de circuito cerrado permiten que la ubicación de las instalaciones sea más flexible. Con ellas se previene la contaminación externa y se reduce la huella ecológica del agua. Sin embargo, actualmente entrañan un elevado coste económico pues requieren una mejora importante de la eficiencia energética para poder competir con los cultivos de circuito abierto. En cuanto a las granjas situadas en costas expuestas, la contaminación del agua de entrada se evita debido a que el elevado hidrodinamismo de las zonas donde se encuentran diluye y dispersa rápidamente el vertido. Sin embargo, dispersar los contaminantes no implica evitar sus efectos, y por lo tanto, la dilución no es la solución.

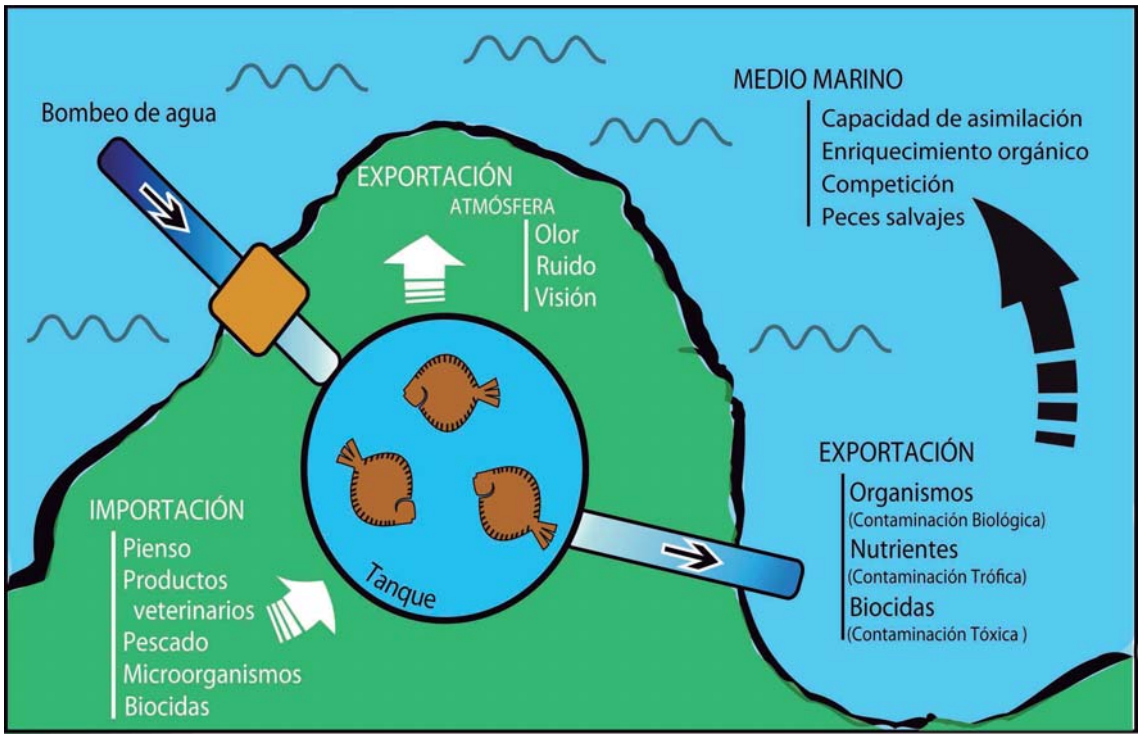


Figura 2. Representación del balance de materiales de una piscifactoría marina instalada en tierra (LBMFF).

1.3.1. Granjas terrestres VS Granjas marinas

Todas las piscifactorías muestran una serie de características comunes, como son la formulación de piensos, la aplicación de sustancias agroquímicas, antibióticos y otros productos, resultando en el vertido de sustancias contaminantes similares.

El cultivo en jaula es el tipo de piscicultura marina más empleada en Europa y en el mundo (Halwart et al., 2007) debido al menor coste de sus instalaciones, aunque este tipo de instalaciones también está expuesto a mayor número de agentes externos (enfermedades externas, rotura de redes por depredadores, temporales, robos,…).

A diferencia de las granjas en tierra, las jaulas no poseen tantas limitaciones territoriales, pues no necesitan encontrarse adyacentes a la costa. Las condiciones de higiene del cultivo requieren de menos cuidados debido a las menores densidades de cultivo y a la continua renovación del agua a través de las redes. Por otra parte, el aporte de materia orgánica al medio marino es mayor por un menor control del contenido orgánico e inorgánico del vertido y un menor aprovechamiento de los piensos, que puede llegar a crear graves cúmulos de desechos debajo de las jaulas. Sin embargo, aunque el vertido de las granjas instaladas en tierra sea menor que el de las jaulas (en volumen, no en toxicidad), al producirse de manera concentrada a través de un único emisario sus efectos ambientales pueden ser más perjudiciales y/o evidentes.

Debido a la baja producción relativa global de los cultivos intensivos en tierra, las instalaciones objeto de estudio de esta tesis pasan bastante desapercibidas, lo cual explica el que sean muy escasos los estudios ambientales sobre este tipo de instalaciones y que no se hayan desarrollado adecuados controles legales para evitar el impacto ambiental que puedan generar.

1.3.2. Restricciones sobre la matriz de estudio y el tipo de análisis

Las evaluaciones del impacto ambiental asociado a la piscicultura marina se han realizado fundamentalmente en jaulas marinas. Estos estudios se centran principalmente en el análisis del sustrato blando o sedimento, ya sea para medir los niveles de contaminantes, su disponibilidad o los cambios

de las comunidades bentónicas. El sedimento y la comunidad bentónica asociada son, por lo tanto, el soporte básico o matriz de estudio comúnmente empleado.

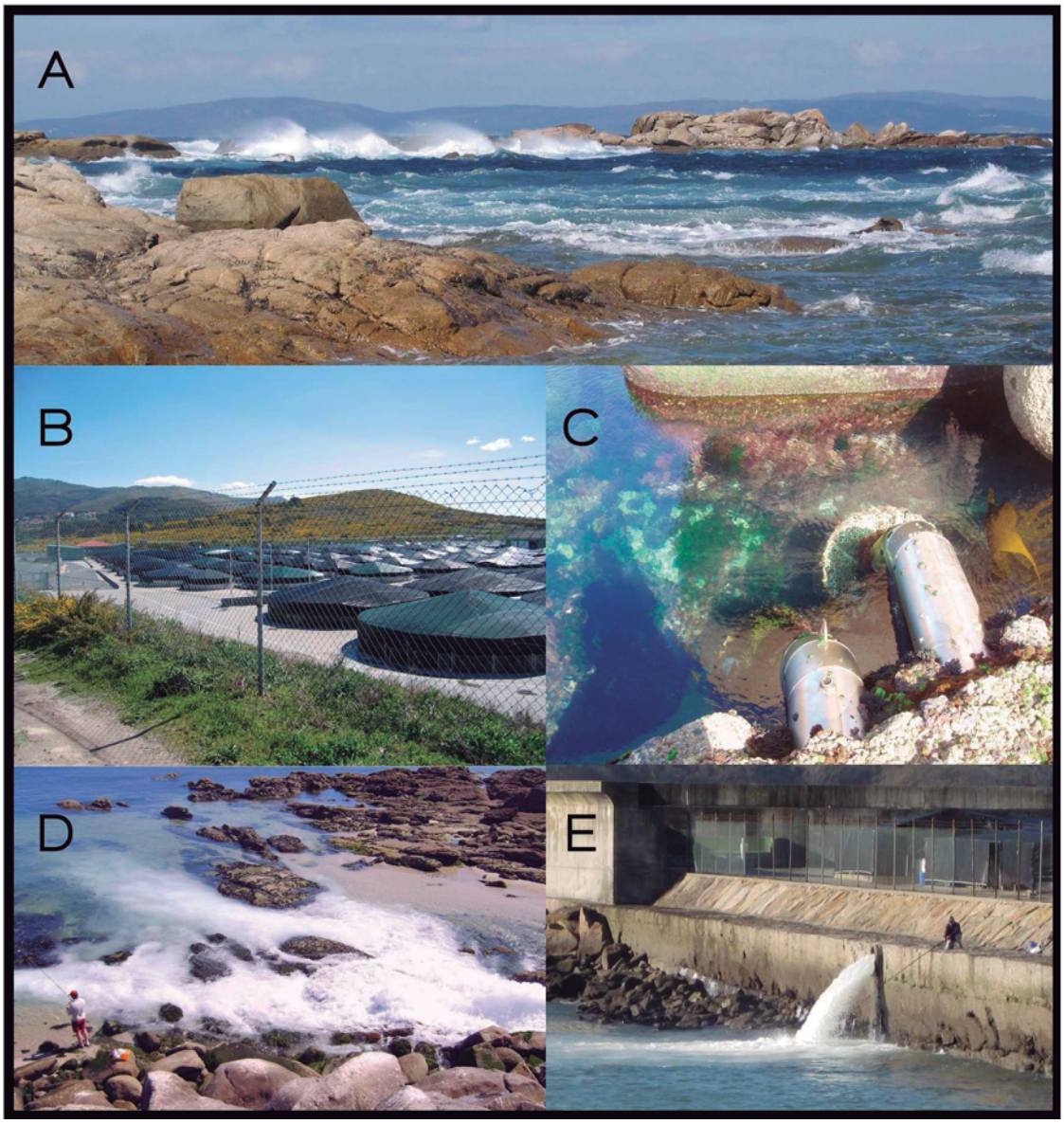


Figura 3. Fotografías características del cultivo intensivo en tierra. **A.** Elevado hidrodinamismo. **B.** Tanques de cultivo. **C.** Toma de agua. **D-E.** Emisario.

Las granjas marinas en tierra han de situarse en lugares expuestos donde el elevado hidrodinamismo dispersa rápidamente los vertidos, pero también las partículas finas del fondo (Figura 3A). De esta manera, los fondos marinos donde estas granjas vierten son, en su mayoría, fondos rocosos- bloques con arenas y gravas-, mientras que la fracción útil comúnmente utilizada para los análisis del sedimento corresponde a las partículas con un diámetro inferior a 63 µm (limos y arcillas) (Casado, 2006). Esta afirmación quedó

confirmada durante el desarrollo de esta tesis al realizar pruebas de tamizado con distintas muestras del fondo marino cercano a las piscifactorías y observarse que después de un oneroso tamizado el porcentaje de fracción útil era despreciable. Por este motivo, se tuvieron que buscar matrices de estudio alternativas: el efluente o vertido directamente y la columna de agua en las inmediaciones de las granjas.

La rápida y elevada dilución del vertido en las zonas de estudio dificultó la detección de cambios significativos mediante el análisis químico convencional de las muestras de la columna de agua ya que todos los contaminantes se encontraban por debajo de su límite de detección. La manera más segura de detectar concentraciones significativas de algún contaminante sería analizando la muestra previa a su dilución (efluente). Sin embargo, esta vía fue descartada, porque el análisis químico de los vertidos también resultó ser una herramienta poco sensible, debido al elevado caudal bombeado.

Además de la baja concentración de contaminantes, es necesario conocer qué sustancias se encuentran en el vertido previo a su análisis, lo cual no es fácil debido a la disparidad de sustancias y la escasa información pública disponible (Sapkota et al., 2008; Burridge et al., 2010).

Después de una amplia revisión bibliográfica se escogió el vertido (para la realización de bioensayos) y el análisis de organismos nativos y trasplantados como la mejor o única forma de estudio del impacto ambiental que puedan generar las granjas marinas intensivas en tierra.

1.4. Características de los efluentes

Los efectos de la piscicultura son reconocidos a nivel global como poco contaminantes cuando los comparamos con otras actividades industriales. Sin embargo, los volúmenes de residuos descargados por dicha actividad también son mucho mayores, llegando a convertirse en auténticos ríos de deshechos. Asimismo, los vertidos actúan como un peligroso dispositivo de concentración de peces silvestres y si los residuos son liberados en exceso pueden producir la degradación del medio por eutrofización, anoxia o toxicidad (Figura 4).

Los efectos potenciales de una granja piscícola sobre el ambiente acuático son debidos principalmente a los residuos contenidos en sus efluentes: heces y productos de excreción, alimento no consumido y productos químicos derivados de las diferentes actividades de la granja.

1.4.1. Contaminación por productos químicos

El uso de productos químicos en la acuicultura está más que reconocido. Aun así, la mayoría de los estudios sobre los efluentes de las granjas en tierra o en mar se han centrado principalmente en la contaminación orgánica bruta, y hay pocos estudios sobre productos químicos y agentes patógenos (Tello et al., 2010). La directiva Marco del Agua de la UE reconoce los contaminantes emergentes como nuevas formas de contaminación pero todavía no se han definido normas ni niveles de referencia al respecto. En consecuencia, el uso de productos químicos, como desinfectantes, antibióticos, pesticidas y hormonas, suele llevarse a cabo de forma libre y por personal que en muchos casos puede no estar debidamente cualificado.

A partir de la información contenida en las publicaciones de la GESAMP (Joint Group of experts on the Scientific Aspects of Marine Environmental Protection) se ha podido realizar un estudio exhaustivo de los principales productos químicos utilizados en la piscicultura de *P. maxima* (GESAMP, 1996) y han podido clasificarse en 7 categorías en función de su mecanismo de acción o procedencia:

- Químicos que proceden de materiales estructurales
- Desinfectantes
- Medicinas
- Pesticidas
- Aditivos de la comida
- Anestésicos
- Hormonas

Entre las distintas funciones que desempeñan estos productos se encuentran el transporte de organismos vivos, el control de crecimiento, la producción de alimentos, la manipulación de la reproducción, la

prevención (*uso profiláctico*) y el tratamiento (*uso terapéutico*) de enfermedades y patógenos o la limpieza de las instalaciones

1.4.1.1. Antibióticos y pesticidas

El empleo de quimioterapia para mantener condiciones sanitarias óptimas en las piscifactorías es inevitable puesto que las medidas alternativas, tales como vacunas, inmunoestimulantes o resistencia genética, están limitadas por cuestiones técnico-económicas (muchas se encuentran aún en proceso de desarrollo) y/o por una eficacia limitada (Tabla 1). En la actualidad, se ha verificado el uso de al menos 26 medicamentos concretos por parte de los 15 principales países productores, que conjuntamente generan el 94% de la producción de acuicultura mundial, siendo la oxitetraciclina uno de los más usados (Campbell et al., 2001).

Tabla 1. Enfermedades más frecuentes del cultivo de rodaballo, organismos que las provocan y medidas para su control.

Enfermedad	Organismo	Medidas de control
Enf. amébica de las agallas	Ectoparásito	Baño de agua dulce
Tricodiniasis	Ectoparásito	Baño desinfectante
Escuticociliatosis	Ecto, Endoparásito	Reducción de densidad
Microsporidiosis	Endoparásito	Reducción de densidad
Mixosporidiosis	Endoparásito	Red. densidad/ Desinfección completa
Flexibacteriosis	Bacteria	Vacuna/antibióticos
Furunculosis	Bacteria	Vacuna/antibióticos
Streptococcosis	Bacteria	Vacuna
Vibriosis	Bacteria	Vacuna/antibióticos

La utilización de antibióticos y pesticidas comporta un riesgo para el medio ambiente, pues una parte de estas sustancias no son retenidas por los peces (antibióticos no metabolizados, restos de piensos medicados, lixiviados, excreción renal y secreciones branquiales) y grandes cantidades entran a formar parte de los efluentes que son vertidos a los sistemas acuáticos (Rigos et al., 2004).

El impacto ambiental de las sustancias terapéuticas no es fácil de determinar y su persistencia y la de sus metabolitos es desconocida y no ha recibido la necesaria consideración (Tello et al., 2010; GESAMP, 1997). Sí se ha estudiado el efecto de los antibióticos sobre comunidades naturales de bacterias, identificándose bacterias con elevadas tasas de resistencia a antibióticos en estanques (Twiddy and Reilly, 1995).

1.4.1.2. Desinfectantes y detergentes

Utilizados por higiene, para prevenir infecciones y puntualmente también para combatir algunas enfermedades, los desinfectantes y detergentes son productos que deben ser considerados por su uso cotidiano en concentraciones relativamente elevadas. Entre los desinfectantes más utilizados están los productos clorados, aldehídos y sus derivados. Estos compuestos son cancerígenos a pesar de que tienen una larga historia de un uso seguro y beneficioso en la desinfección del agua potable (Corporation Black and Veatch, 2010).

El hipoclorito de sodio es un oxidante fuerte y económico mundialmente usado, que se aprovecha por sus propiedades desinfectantes del agua, tanques y equipo. Este compuesto es tóxico para la vida acuática en muy bajas concentraciones y oxida la materia orgánica generando una mezcla de compuestos organoclorados que incluyen substancias mutagénicas y cancerígenas (Corporation Black and Veatch, 2010).

Las soluciones de formaldehído son tóxicas para ciertas especies acuáticas, desde plancton a peces, a altas concentraciones o tras períodos de exposición largos (Chénier, 2003).

La toxicidad de los detergentes depende del tipo: aniónicos, no-iónicos y catiónicos (Conti, 1987). A baja concentración pueden modificar la

estructura de las proteínas de membrana y son responsables de la progresiva permeabilización y lisis celular

El efecto tóxico combinado de determinados desinfectantes y detergentes puede ser sinérgico (Panouillères et al., 2007). Por esta razón, deberían realizarse estudios ecotoxicológicos de estos compuestos considerándolos aisladamente o en mezclas.

1.4.2. Contaminación por residuos metabólicos y pienso excedente

Una granja piscícola puede verter determinados productos químicos, alterar las características físico-químicas del medio receptor (Tª, pH, salinidad,...), pero el impacto ambiental habitualmente reconocido deriva de los desechos metabólicos (nitratos, nitritos, amonio y fosfatos) y los restos de comida.

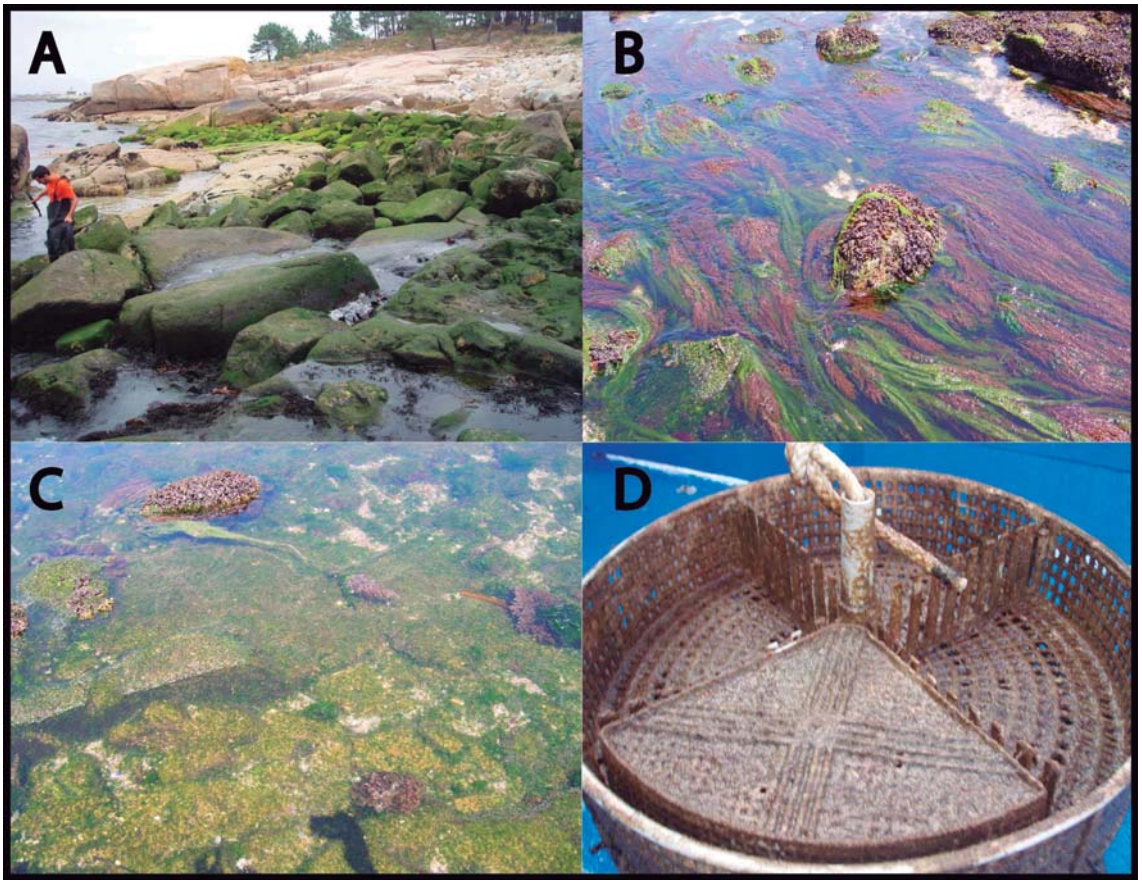


Figura 4. Fotografías de lugares eutrofizados y cercanos al punto de descarga de los vertidos (**A, B** y **C**) y de una jaula para bioensayos después de 45 días de exposición a los vertidos (**D**).

Un impacto muy común de los vertidos orgánicos consiste en crear situaciones de anoxia sedimentaria y potenciar la eutrofización del medio receptor (Figura 4). Mientras los vertidos episódicos locales no son considerados una amenaza para la salud del ecosistema, los crónicos y extensivos pueden producir un deterioro indeseable. En este sentido, algunas granjas piscícolas instaladas en tierra, según sea su relación *producción/capacidad dispersiva del medio*, podrían desarrollar perturbaciones crónicas más o menos graves.

Recientemente se ha elaborado en el Centro Tecnológico Gallego de Acuicultura un estudio que pretende minimizar los residuos de las granjas de *P. maxima* (CETGA, 2005). En este estudio se estableció una media actual de ingestión de pienso del 77%, con un mínimo y un máximo del 27 y 100%, de tal forma que un 23% del pienso suministrado, se estima, es expulsado al medio. En el mismo estudio se obtuvo una media de heces (alimento no digerido) del 13,5%, con mínimos y máximos del 9 y 26%. De esta manera, el 63,5% del pienso pasó a ser asimilado o fue excretado principalmente como amonio, y el 36,5% restante fue expulsado al medio como pienso y heces.

1.4.2.1. Residuos disueltos

Los efectos de los residuos disueltos sobre la columna de agua son muy difíciles de observar (Sarà, 2007). La dilución del *amonio*, el principal producto de excreción disuelto de los peces, es tan rápida que es difícil medir trazas de este elemento incluso a poca distancia de las granjas (Pitta et al., 2005; Pitta et al., 2009). Sin embargo, la disponibilidad permanente de amonio en la proximidad de los emisarios puede generar un proceso de eutrofización crónico capaz de aumentar el epifitismo y el desarrollo de *fouling* sobre estructuras, alterar la comunidad algal, entre otros efectos.

Según varios estudios con *P. maxima* (Dosdat et al., 1996; Fournier et al., 2003) la excreción de urea representa el 20% de la excreción total de nitrógeno y existe un debate acerca de si la urea se transforma en amonio durante la retención y manipulación del agua (Aubin et al., 2006) o si no existen cambios significativos en las concentraciones (Altinok and Grizzle, 2004).

1.4.2.2. Sólidos en suspensión

Las partículas orgánicas procedentes de las heces y de alimento no consumido pueden almacenarse en el sedimento y si no son debidamente mineralizados por la macrofauna y la flora bacteriana natural pueden llegar a acumularse y degradar el ecosistema bentónico (Sanz-Lázaro et al., 2011). Sin embargo, el alto grado de hidrodinamismo de los sitios donde se localizan las granjas intensivas instaladas en tierra y la baja cantidad de alimento no consumido que liberan (cuya regulación supone un ahorro económico para la propia empresa) en comparación con las jaulas dificultan la acumulación de la materia orgánica en el entorno del emisario. Se ha comprobado que, en general, tanto el porcentaje de la fracción fina como de la materia orgánica del sedimento son muy bajos en el entorno de las granjas instaladas en la costa de Galicia pero sin embargo, es fácil observar una elevada y continua presencia de sólidos en suspensión en la columna de agua en las inmediaciones de los emisarios.

1.4.3. Otros tipos de contaminación

El riesgo de contaminación biológica (introducción de especies exóticas y patógenos) y el riesgo de polución genética (intercambio genético entre peces de granjas y especies nativas) que afecten a las poblaciones silvestres del entorno se considera muy bajo en este tipo de instalaciones de cultivo frente al de jaulas.

1.5. Granjas y Zonas de estudio

Se seleccionaron 8 granjas terrestres de *P. maxima* del Noroeste peninsular, cuya producción corresponde al 50% de la producción española de esta especie (APROMAR, 2011) (Figura 5). A excepción de la granja VIII, que es de circuito cerrado, todas las demás granjas presentan un sistema de cultivo en circuito abierto. Existen diferencias significativas entre las granjas en cuanto a la producción y el correspondiente volumen de residuos que generan (Figura 1).

Todas las granjas se encuentran situadas a escasos metros sobre el nivel de mar, para evitar gastos innecesarios de bombeo de agua, y la mayor parte de ellas se encuentran situadas en costas muy expuestas (excepto las granjas I y VIII).

Con el fin de evitar la contaminación del agua de entrada las granjas se encuentran alejadas de otros focos de contaminación, aparte de algún pequeño núcleo residencial, y excepto la granja I, ubicada cerca de una industria de aluminio de gran magnitud. Gracias a la escasa o nula influencia de otras fuentes de contaminación cercanas, se asume que el estado ambiental en las cercanías de las granjas es responsabilidad y específico de éstas. La ausencia de interferencias con otros focos de contaminación facilita la puesta en evidencia de los efectos propios de esta actividad.

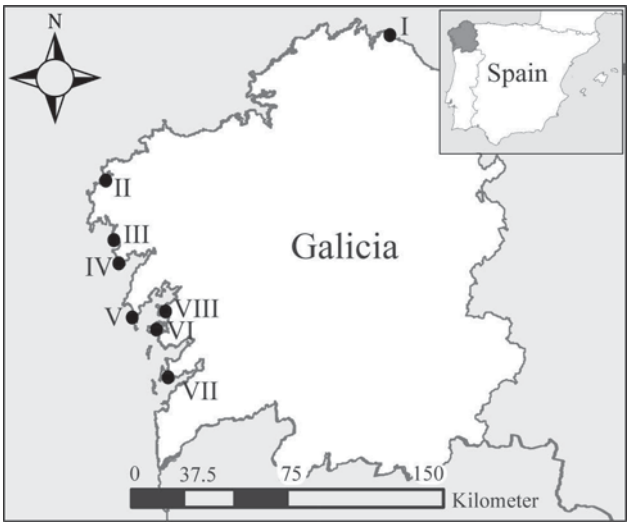


Figura 5. Localización de las piscifactorías marinas instaladas en tierra (LBMFF) estudiadas en esta tesis.

1.6. Sostenibilidad de la acuicultura

1.6.1. Principios y objetivos internacionales

La (UNEP, 2011) en su último informe propone una serie de medidas respecto a la acuicultura:

- *Establecer un plan ambiental para asegurar la mínima degradación ambiental*
- *Parar la producción de peces carnívoros hasta que existan fuentes de alimento para los peces alternativas a la explotación pesquera*
- *Adoptar tecnologías integradas que sean autónomas y autosuficientes*

- *Desarrollar sistemas de gestión que desemboquen en actividades respetuosas con el medio*

Para poder establecer un PVA que asegure el mínimo impacto en el ecosistema es necesario que la piscicultura sea sostenible y, por lo tanto, ha de estar integrada en el área y en las poblaciones donde está localizada. Con este fin, la (FAO, 2010) ha desarrollado una serie de principios básicos:

- *El desarrollo y la gestión de la acuicultura ha de tener en cuenta todas las funciones y servicios del ecosistema. Esto implica definir los límites del ecosistema mediante el estudio de su capacidad de asimilación y de carga y la futura adaptación de las instalaciones acuícolas.*
- *La acuicultura ha de aumentar el bienestar del hombre y garantizar la igualdad entre las partes involucradas*
- *La acuicultura ha de seguir un desarrollo integrado e interactuar con otros sectores de producción para promocionar el reciclado de materiales y hacer un uso óptimo de los recursos*

1.6.2. Vigilancia ambiental actual

El Plan Sectorial de Acuicultura Terrestre de Galicia pretendía alcanzar una producción de 30.000 toneladas de rodaballo al año a través de granjas marinas intensivas instaladas en tierra, para las que los planes de vigilancia ambiental (PVA) son prácticamente inexistentes.

Según la ley 23/1984, de 25 de Junio, de cultivos marinos, el Ministerio de Agricultura, Pesca y Alimentación puede proponer planes nacionales de cultivo, delegando la responsabilidad de éstos sobre las comunidades autónomas. Augas de Galicia, es el organismo responsable de la vigilancia ambiental de ríos y costas gallegas y aplica a las piscifactorías un Control basado en normas sobre Calidad del Vertido. Este control se centra en regular los incrementos máximos autorizados de: sólidos en suspensión, nitritos, fosfatos y carbono orgánico total, vertidos al mar en relación con las aguas de entrada a la granja marina (Tabla 2).

Tabla 2. Incrementos máximos autorizados de vertido al mar en relación con las aguas de entrada a la granja marina (Augas de Galicia, XUGA).

Parámetro	Incremento (SalidaÆEntrada)	Periodicidad
Sólidos en Suspensión	<5 mg/L	Trimestral
Nitritos	<0.05 mg/L	Trimestral
Fosfatos	<0.2 mg/L	Trimestral
Carbono Orgánico Total	<0.5 mg/L	Trimestral

Este control de vertido parece ser poco exigente, tanto en los límites de emisión como en la frecuencia de muestreo, si lo comparamos, por ejemplo, con las normas aplicables al tratamiento de las aguas residuales urbanas (RD 509/1996).

En cuanto al control del medio receptor, Augas de Galicia, selecciona cinco puntos de muestreo de agua:

- En la salida directa en la superficie de las aguas
- A 50 m del foco siguiendo la dirección de las corrientes dominantes
- En la zona marisquera más próxima
- En la zona de baño más próxima
- En la zona de no afección, que será considerado como Valor de Fondo

El control del medio receptor se realiza anualmente, y en este caso se añade la determinación de nitrógeno amoniacal a los cuatro parámetros de verificación del vertido. Los parámetros descritos para el control del medio receptor no se adecuan a todo tipo de explotaciones. Estos controles están basados en escasos parámetros que se ven fuertemente afectados por las condiciones hidrodinámicas del día de muestreo y por la elevada dilución de la contaminación. Además en ninguno de los controles se consideran los posibles efectos tóxicos del vertido sobre la biota nativa.

1.7. Hipótesis y objetivos de esta tesis

Esta tesis pretende suministrar el conocimiento necesario para un PVA y responder al primero de los principios de la UNEP para el caso concreto de las granjas marinas intensivas terrestres instaladas en costas expuestas con fondos rocosos. La falta de conocimientos previos y modelos para predecir riesgos ecológicos asociados a este tipo de instalaciones nos conduce a la necesidad de explorar diferentes perspectivas y herramientas metodológicas que pudieran servir como base científica para el diseño de un PVA integrado y dinámico.

Desde una perspectiva ecotoxicológica, la vigilancia del posible impacto que pueden ocasionar los vertidos requiere de la medición de múltiples parámetros, que adquiridos de manera adecuada y en cantidad suficiente permitan extraer conclusiones robustas a través de un análisis estadístico correcto (Carballeira et al., 2012d). Desde un punto de vista económico, solamente se tendrán en cuenta aquellos parámetros que sean relevantes para el seguimiento, valorando la relación entre el coste de adquisición y la información que aportan a la vigilancia (Borja, 2002).

De esta forma, los Planes de Vigilancia Ambiental (PVA) surgen como un seguimiento de forma estandarizada y a su vez ajustada a una serie de condiciones locales que permitan anticiparse a un impacto crónico mediante el establecimiento de controles periódicos de los parámetros de mayor importancia.

Objetivos

- Seleccionar y confirmar los parámetros indicadores y las metodologías aplicables al estudio y análisis de los parámetros propuestos.
- Definir y determinar los valores de referencia para dichos parámetros.
- Elaborar relaciones causa-efecto para la correcta monitorización ambiental de la acuicultura marina en tierra.
- Generar un protocolo para la formulación de Programas de Vigilancia Ambiental especialmente adaptados a las granjas marinas en tierra.

Hipótesis

¿Es la acuicultura marina intensiva e instalada en tierra respetuosa con el medio?

Y en el supuesto caso de que las granjas no se encuentren completamente integradas, ¿Qué herramientas que permitan detectar el impacto de estas granjas en el medio se deberían incluir en su plan de vigilancia ambiental?

1.8. Estructura de esta tesis

En primer lugar, se realizó una revisión bibliográfica de todas las herramientas y métodos que podrían ajustarse a las condiciones especiales de este tipo de granjas, orientada hacia la elaboración de un PVA. Esta tesis se presenta en varios capítulos seleccionados a partir de la aproximación conceptual del peso de la evidencia (Chapman, 2007; DelValls, 2007). Los distintos subapartados fueron adoptados a partir de la información contenida en la revisión bibliográfica y de los propios resultados que se iban obteniendo durante la realización de esta tesis.

HERRAMIENTAS FUNDAMENTALES DE UN PLAN DE VIGILANCIA AMBIENTAL

2.1. Metodología integrada

Para obtener una idea comprensiva del conjunto de relaciones establecidas entre los parámetros de un PVA es conveniente contar con una aproximación conceptual. Para este fin se puede considerar la aplicación de investigaciones basadas en "el peso de la evidencia" (WOE, *Weight Of Evidence*) que nos permite determinar posibles impactos ecológicos debido a un conjunto de estresores fundamentados en varias "líneas de evidencia" (LOE, *Lines Of Evidence*) (Chapman, 2007).

El análisis químico informa de los contaminantes presentes en el medio pero no indica de qué forma se ve afectada la biota (biodisponibilidad, bioacumulación, cambios en las comunidades nativas,...) (Borgmann et al., 2001; DelValls, 2007). La aproximación WOE permite integrar de forma sencilla las relaciones que estos contaminantes tienen con el medio que los rodea. La principal ventaja de estos métodos es la integración de resultados de muy distinta naturaleza; desde la degradación físico-química del medio hasta la alteración de las comunidades biológicas receptoras.

Figura 6. Esquema conceptual del método integrado (TRIAD) aplicado en esta tesis. El método integrado consta de tres líneas de evidencia (LOEs) y está basado en la aproximación del peso de la evidencia (WOE).

Esta aproximación es flexible y permite añadir o cambiar los ejes que lo forman o los estudios que integran cada uno de estos ejes, siempre que la integración de las líneas de evidencia permitan describir los criterios de

calidad ambiental a los que se encuentra sometido el medio circundante (área de solapamiento de las líneas).

Aunque está aproximación se ha utilizado principalmente para evaluar la calidad de los sedimentos (SQT, Sediment Quality Triad) (Caeiro, 2004; DelValls and Chapman, 1998; Morales-Caselles et al., 2009) es apropiado recurrir a ésta para la explicación de la relación foco de contaminación y la extensión del impacto.

Para una vigilancia integral de estas granjas hemos organizado y combinado los estudios en 3 grandes ejes o líneas de evidencia: *Exposición a los contaminantes* (mediante *factores de contaminación* que relacionan las concentraciones reales con las de *fondo*, tanto en medio como en organismos), *Alteración específica* (como afectan estas concentraciones a los organismos, *in situ* y en laboratorio) e *Indicadores de Integridad Ecológica* (informan sobre la alteración de la estructura y el comportamiento de las poblaciones o comunidades nativas) (Figura 6).

2.1.1. Exposición a los contaminantes

Para la determinación del grado de exposición al que están sometidos los organismos del medio es necesario medir las características físico-químicas y las concentraciones de los contaminantes en las matrices disponibles en dicho medio. En estas áreas, debido al elevado hidrodinamismo, las determinaciones se tuvieron que realizar en el vertido (previo a su dilución en el medio) y en organismos nativos. Mediante el uso de una sonda multiparamétrica, espectrofotómetro de absorción atómica con cámara de grafito, espectrofotómetro de absorción atómica de llama y espectrómetro de masas isotópicas se obtendrían las características físico-químicas del vertido y las concentraciones de los contaminantes más destacables presentes en los vertidos y organismos.

Dentro de esta línea de evidencia cabe destacar por su relevancia una serie de conceptos o herramientas metodológicas:

2.1.1.1. Área de influencia y toxicidad potencial

El *área de influencia* viene determinada por el alcance máximo de los efectos ambientales generados por los vertidos. La *toxicidad potencial*, presente dentro del área de influencia, describe la capacidad que tienen los contaminantes de ser incorporados por los organismos y provocar un cambio perjudicial. Estos parámetros han de ser definidos a través de las diferencias en los parámetros físico-químicos del agua de entrada y salida (*toxicidad potencial*) y la acumulación de sustancias de origen antropogénico en biomonitores localizados a distinta distancia del emisario siguiendo la corriente predominante (*área de influencia*) (Carballeira et al., 2012d; Rey-Asensio et al., 2010).

El muestreo de organismos nativos permite, además de determinar medidas de exposición, realizar estudios asociados a la integridad ecológica. Además, los parámetros pertenecientes a cada LOE, seleccionados de forma apropiada, pueden ser correlacionados facilitando los análisis de integridad ecológica mediante sencillas medidas de análisis químico (ej. señal isotópica δN^{15}-estudios histopatológicos) (Carballeira et al., 2011b).

2.1.1.2. Señal isotópica $\delta^{15}N$

De manera natural se encuentran dos formas de Nitrógeno en el medio; la más común contiene siete protones y siete neutrones (^{14}N) y la otra forma contiene un neutrón extra (^{15}N). Estos isótopos se producen en una proporción constante en la atmósfera, que es utilizada como estándar (Nier, 1950; Robinson, 2001). Esta proporción varía en función de las vías metabólicas y de las diversas reacciones del ciclo del N que la molécula sigue.

Las diferentes fuentes de contaminación costera (agricultura, urbana,…) presentan relaciones isotópicas singulares, lo que permite su identificación (ej. para los residuos urbanos es de $10^{0}/_{00}$). Las algas absorben y asimilan el N procedente de las aguas residuales, lo cual se refleja en la δN^{15} de sus tejidos. Al no existir selectividad entre los isótopos, ^{15}N y ^{14}N, y que la señal aumenta con el suministro de N (ya que adquieren más rápidamente el

$^{15}NH^{+}_{4}$), las variaciones de δN^{15} en las algas recolectadas o trasplantadas en el entorno de un vertido permiten detectar y observar la extensión del N biodisponible (Deutsch and Voss, 2006; Lin and Fong, 2008; Savage and Elmgren, 2004).

La señal isotópica δN^{15} se obtiene al determinar la *relación isotópica* $^{15}N/^{14}N$ ($R_{problema}$), tanto en muestras bióticas como abióticas (Savage, 2005), y compararla con la del aire ($R_{estándar}$):

$$\delta N^{15}\left({}^{0}/_{00}\right) = (R_{problema} / R_{estándar} - 1) \times 10^{3}$$

La señal isotópica $\delta^{15}N$ ha sido previamente utilizada con éxito como trazador de los efluentes nitrogenados liberados por granjas de peces (Costanzo et al., 2004; Dolenec et al., 2007; Jones et al., 2001; Lin and Fong, 2008; Lojen et al., 2005; Sarà et al., 2004). En este sentido, la relación isotópica $\delta^{15}N$ es un magnífico descriptor de exposición ya que integra el binomio: *Carga contaminante-Capacidad dispersiva del medio*.

Se ha demostrado que las especies del género *Fucus* sp., algas marrones ampliamente extendidas en las costas gallegas, son excelentes biomonitores para detectar cambios de origen antropogénico en la señal isotópica $\delta^{15}N$ (Riera et al., 2000; Savage and Elmgren, 2004).

2.1.2. Alteración específica

Los efectos potenciales de los residuos piscícolas se pueden clasificar en dos grandes grupos: Efectos Tróficos y Efectos Tóxicos. Aquellos organismos cuyo desarrollo dependa, directa o indirectamente, de la presencia de nutrientes en el medio pueden verse afectados por ambos efectos. Así, el efecto de los residuos sobre el desarrollo algal va a ser el producto combinado de la presencia de inhibidores del crecimiento (tóxicos) y de activadores del crecimiento (nutrientes). Dentro del esquema triaxial se pueden reconocer claras interacciones entre descriptores pertenecientes a distintas líneas de evidencia (p.e. *bioacumulación de contaminantes-biomarcadores de efecto; relación isotópica $\delta^{15}N$-eutrofización*;...), lo que refuerza la

información obtenida y permite establecer relaciones de intercalibrado *exposición-efecto* que pueden facilitar la vigilancia ambiental.

La determinación analítica de los contaminantes en mezclas complejas de composición desconocida, una situación común en muchos vertidos, no permite una estimación relevante de la toxicidad o de los efectos tróficos. Por ello, la exposición de los organismos a estos vertidos es generalmente conocido como el mejor método para evaluar los efectos tóxicos y tróficos (Pandard et al., 2006). Esta herramienta básica se conoce como *bioensayo*.

2.1.2.1. Bioensayos

Desde los años 1970s se han desarrollado numerosos bioensayos para estudiar los efectos de los contaminantes en el medio acuático (Selck et al., 2002). Actualmente son numerosísimas las técnicas de bioensayos, desarrolladas y estandarizadas en mayor o menor grado, para aplicar en programas de biomonitorización marina (Hylland, 2001; ICES, 2002).

La principal ventaja de la realización de bioensayos es la integración de todos los efectos de los contaminantes, incluidos los aditivos, los sinérgicos y los antagónicos. Además, proporcionan información valiosa sobre la fracción biodisponible de todos los contaminantes, y no sólo los que se detectan, *a priori*, mediante análisis químico (Cairns et al., 1975; Carballeira et al., 2012a).

Como no todas las especies o fases del ciclo vital son igual de sensibles es imprescindible identificar que especies test serán potencialmente afectadas por el tipo de compuestos que se emiten. La elección de dichas especies estará determinada por su relevancia, prevalencia, accesibilidad, simplicidad de mantenimiento y cultivo, bajo coste, y facilidad en la observación y cuantificación de los efectos.

En este sentido, y para intentar detectar los efectos de todos los contaminantes presentes es conveniente considerar una batería de bioensayos de, al menos, tres organismos pertenecientes a distintos niveles tróficos. Según (Peters et al., 2002) los criterios para seleccionar las distintas especies marinas que formarán la batería mínima de bioensayos son:

- Las especies deben ser nativas y representar diferentes taxones y niveles tróficos, para cubrir las diferentes vías de entrada de los tóxicos

- Las especies deben ser sensibles a un amplio espectro de contaminantes

- Los tests deben estar estandarizados, ser prácticos y los costes adecuados a los resultados.

Por lo general, los bioensayos de toxicidad más utilizados en medio marino se realizan con microorganismos y fases tempranas del ciclo vital de invertebrados. Los organismos de pequeño tamaño o fases tempranas son hasta 3 órdenes más sensibles que los de gran tamaño o fases adultas porque su elevada relación superficie/volumen permite a éstos absorber rápidamente los contaminantes, sufriendo de forma más intensa sus efectos (Persoone et al., 2000).

Los bioensayos pueden ser clasificados según 4 criterios: la aplicación de sus tratamientos, el lugar de desarrollo, los efectos y la duración. Se consideró oportuno diferenciar, dentro de la línea de evidencia de alteración específica, los bioensayos según localización debido a la distinta sensibilidad y realismo de las respuestas.

2.1.2.1.1. Tipos de bioensayos

Bioensayos de laboratorio

Los bioensayos de laboratorio o *in vitro* surgen por la necesidad de estandarizar las evaluaciones ecotoxicológicas de sustancias químicas puras o complejas y toda clase de muestras ambientales, bajo condiciones controladas, lo que permite calcular la relación concentración-efecto existente. Además, la aplicación de una batería de bioensayos en laboratorio usando conjuntamente especies de distintos niveles tróficos está reconocida como una herramienta sencilla y útil que permite determinar la toxicidad de los vertidos de aguas residuales (Helsinki Commission, 2011).

Bioensayos de campo

Los bioensayos de campo o *in situ* representan la toxicidad de una forma más realista al exponer el organismo en el lugar exacto del impacto, pues integran los tests de toxicidad desarrollados en laboratorio, los análisis químicos y las medidas de alteración de la integridad ecológica. También se utilizan para correlacionar los resultados de bioacumulación y el estado de los biomarcadores en condiciones realistas (DelValls, 2007; Martin-Diaz et al., 2005).

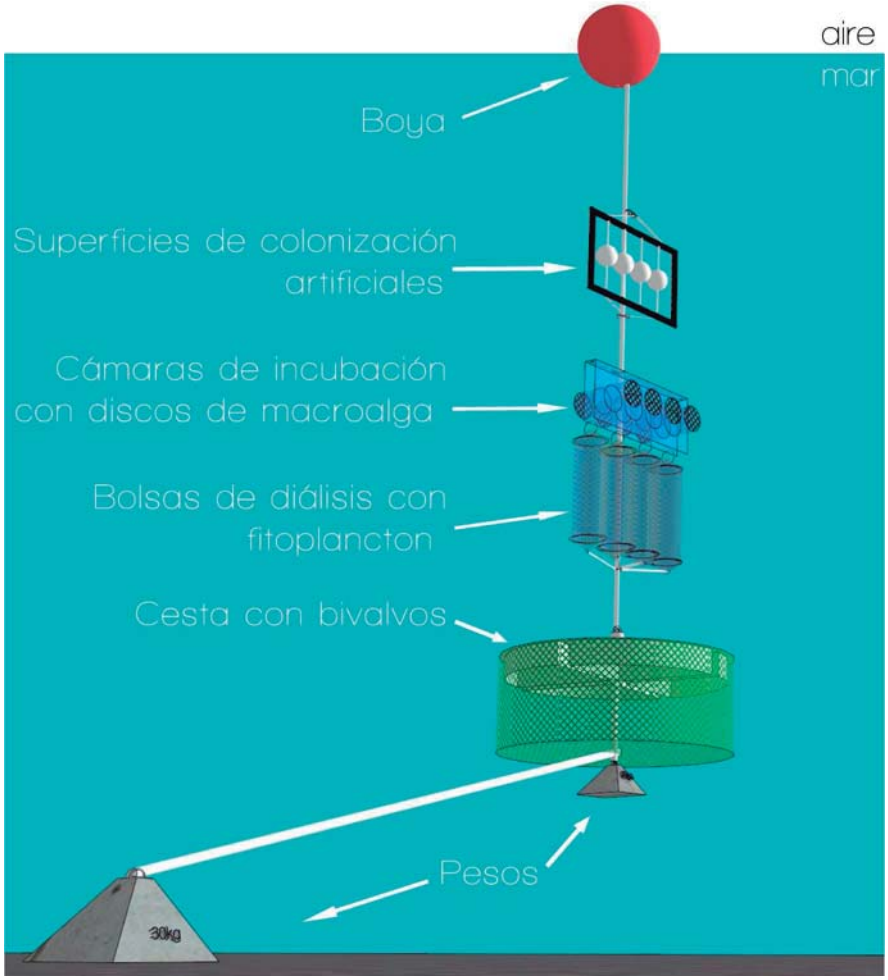


Figura 7. Dispositivo utilizado para la realización de múltiples bioensayos *in situ*.

Entre los bioensayos *in situ* que se pueden plantear en el caso de las granjas piscícolas están los de trasplantes de algas y moluscos nativos o comerciales. Este tipo de bioensayos son fáciles de desarrollar y además suministran información sobre la viabilidad de instalar cultivos multitróficos

asociados a los vertidos de las granjas, con la doble finalidad de aumentar su producción y de actuar como dispositivo de depuración.
Con el fin de realizar varios bioensayos *in situ* y de manera simultánea (para que los resultados fueran comparables por presentar similar grado y momento de exposición) con especies de distintos niveles tróficos se creó un dispositivo que pudiese soportar de la forma más estandarizada, segura, práctica y realista todos los organismos expuestos (Figura 7).

2.1.2.1.2. Parámetros ecotoxicológicos

Los valores de NOEC (*No Observed Effect Concentration*) y de LOEC (*Lowest Observed Effect Concentration*) han sido ampliamente utilizados para evaluar la toxicidad de tipo crónico de toda clase de sustancias. Por el tipo de contaminación que generan las piscifactorías, baja concentración y emisiones crónicas, estos parámetros serían los más adecuados para utilizar como criterios de calidad. Sin embargo, la fiabilidad y la exactitud de estos parámetros toxicológicos dependen en gran medida de la gama de concentraciones bioensayadas y del número de réplicas utilizadas (Chapman et al., 1996; Isidori et al., 2005). Un procedimiento alternativo es construir las curvas de toxicidad-respuesta y estimar los criterios de calidad en términos de EC_x (*Effective Concentration*). La EC_x es la concentración de una sustancia que causa una magnitud definida (x) de respuesta en un sistema dado.
La EC_{50} es considerada como uno de los criterios más robustos en los estudios toxicológicos. Sin embargo, no puede ser considerada como un criterio de protección desde una perspectiva ecológica y, además, la baja toxicidad de los efluentes impide el cálculo de este valor en muchos casos.
La NOEC y LOEC indican un grado de calidad imprecisa (variando entre el 5 y 30% respecto al control) mientras que las ECx bajas (p.e. EC_{20}, EC_{10}) arrojan valores similares a las anteriores, son criterios más realistas, estadísticamente más robustos y representan un grado permisible de alteración de la calidad (Kusui and Blaise, 1999).

2.1.2.1.3. Índice PEEP (Potential Ecotoxic Effects Probe)

Este índice integra la toxicidad obtenida en una batería de bioensayos (que representan diferentes niveles tróficos y efectos tóxicos) y la pondera con el volumen del vertido liberado al medio acuático.

El PEEP es adecuado para la evaluación y comparación de los efluentes de las granjas, que presentan una toxicidad relativamente baja, ya que el verdadero impacto en el medio está directamente relacionado con la cantidad que se vierte y que el ecosistema es capaz de asimilar (*capacidad de asimilación*). Éste índice se suele calcular a partir de valores de la NOEC y la LOEC, que no siempre se pueden obtener (Isnard et al., 2001), de modo que el índice se calculó con los valores de EC_{10} y EC_{20}.

Este índice se calcula a través de la siguiente fórmula (Blaise and Férard, 2005):

$$PEEP = \log_{10}\left[1 + n \cdot \left(\sum \frac{T_i}{N}\right) \cdot Q\right]$$

Donde

Ti: *Unidades tóxicas* de cada test (i)

$T = C/EC_x$

C: concentración máxima del efluente

EC_x: EC_{10} o EC_{20}

n: número de bioensayos en los que se observa una respuesta tóxica

N: Número máximo de respuestas medibles;

Q: Caudal del efluente (m^3/h)

$[n \cdot (\sum Ti/N)]$: *Huella tóxica;*

$[n \cdot (\sum Ti/N) \cdot Q]$: *Carga tóxica*

2.1.3. Integridad ecológica

Los índices biocenóticos construidos con la comunidad de macroinvertebrados de la infauna de sustratos blandos son tradicional e históricamente los más utilizados para evaluar cambios en la integridad ecológica o comparar el estado de distintos escenarios del medio marino.

Sin embargo, la inexistencia de sedimento fino en costas fuertemente expuestas obliga a centrar la vigilancia en las comunidades bentónicas sobre sustrato duro de la franja intermareal, por ser potencialmente las más impactadas debido a la disposición en superficie de los emisarios de las granjas. Sin embargo, la caracterización de la composición, estructura y funcionamiento de estas comunidades suele ser compleja, costosa y lenta (DelValls, 2007). Por ello, es muy importante decidir qué tipo de datos tomar que no supongan una pérdida gruesa de información y que se ajusten al procedimiento analítico que se vaya a emplear (Warwick, 1993).

La integridad ecológica puede ser correctamente evaluada, en estos casos, mediante vías alternativas como bioensayos *in situ* de trasplantes de la comunidad de fitoplancton y de colonización de sustratos artificiales (*fouling*) o el estudio de biomarcadores en moluscos nativos o trasplantados (Carballeira et al., 2011b; Carballeira et al., 2012d).

2.1.3.1. Bioensayo de colonización de sustratos artificiales

Una técnica alternativa, más sencilla de cuantificar que los estudios de campo de las comunidades intermareales sobre sustrato rocoso, consiste en vigilar el impacto de las granjas piscícolas mediante el estudio de las comunidades colonizadoras de sustratos artificiales o de superficies naturales, limpias y esterilizadas, localizadas a modo de gradiente de exposición en la zona de influencia de los vertidos (Cook et al., 2006) (Figura 8). Además, las condiciones estandarizadas de exposición de los sustratos artificiales (forma, profundidad, material,…) permiten comparar las alteraciones ecológicas observadas de forma más precisa que las procedentes de los estudios de comunidades nativas.

El estudio de la tasa de renovación específica (Koleff et al., 2003; Wilson and Shmida, 1984) de las comunidades instaladas a lo largo de un gradiente de exposición permite caracterizar el grado y la extensión del impacto en términos de integridad ecológica. Adicionalmente, es un método no destructivo que permite obtener cinéticas de las respuestas a nivel de comunidad.

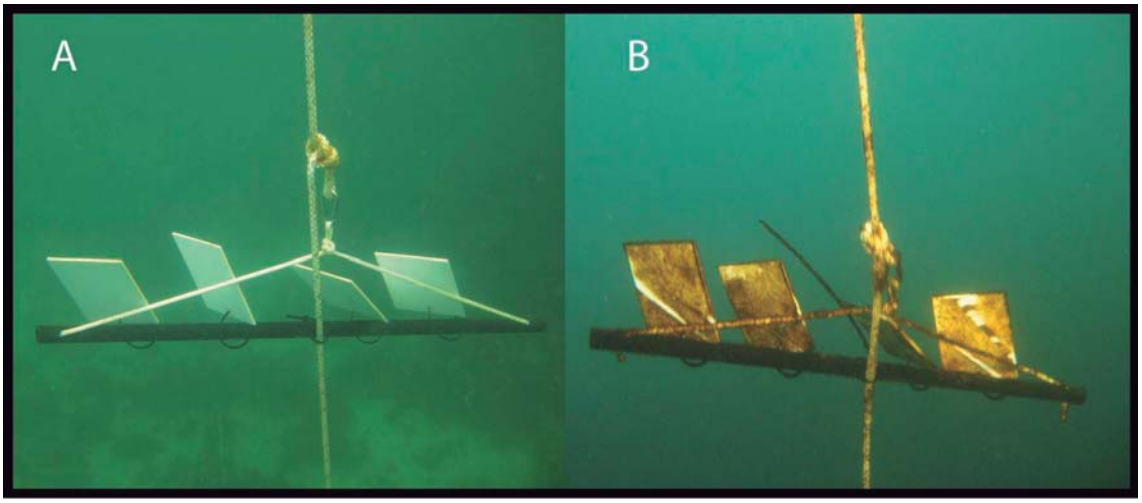


Figura 8. Superficies artificiales de colonización al principio (**A**) y al final del bioensayo (**B**).

2.1.3.2. Bioensayos de fertilidad-toxicidad

La determinación de $\delta^{15}N$ en macroalgas es un parámetro muy práctico pues nos permite, sin dificultad y a bajo coste, detectar el área de influencia y el peso relativo del nitrógeno biodisponible emitido. Pero es necesario tener en cuenta que el valor indicador de la $\delta^{15}N$ en situaciones de contaminación multifocal puede ser dudoso y que no todo enriquecimiento en N se traduce, automáticamente y en cualquier condición ambiental, en crecimiento algal. Por ello, los bioensayos de fertilidad-toxicidad son una herramienta adicional pues suponen un camino intermedio en la vigilancia ambiental entre el análisis químico tisular y las evidencias de degradación *in situ* trófica y tóxica (Carballeira et al., 2010b).

Se pueden realizar bioensayos de fertilidad con macro y microalgas, y en condiciones de laboratorio o de campo. Los parámetros de control pueden ser muy diversos (tasa de absorción de nutrientes; fluorescencia clorofílica; composición pigmentaria; etc.) pero el parámetro más apropiado para caracterizar procesos eutróficos, por integrar la acción conjunta de todos los factores ambientales, es la productividad primaria neta.

En condiciones de laboratorio se pueden realizar los tradicionales ensayos de microalgas con el objeto de evaluar la capacidad trófica de un vertido y de calcular la tasa de dilución necesaria para que no afecte el crecimiento significativamente frente al control.

Los bioensayos de campo nos informan de manera más realista de lo que le ocurre a los productores primarios.

2.1.3.4. Perfiles Ecológicos

Originalmente un perfil ecológico es una serie ordenada de frecuencias de las presencias de una especie (*variable respuesta*) en las clases de una variable del medio o de un descriptor (*variable explicativa*). Los perfiles ecológicos permiten estudiar el grado de asociación de determinadas variables respuesta a lo largo del gradiente de cada variable explicativa. Las variables explicativas han de ser sensibles a los vertidos piscícolas, sencillas de obtener e interpretar y capaces de establecer relaciones de causa-efecto. Para el estudio de la integridad ecológica las variables explicativas seleccionadas pueden ser los parámetros físico-químicos del agua o un sencillo descriptor como es la distancia al foco de emisión y que representa el grado de exposición. Como variables respuesta se pueden utilizar a nivel específico (vigor, frecuencia, abundancia,… de cada especie) o a nivel de la comunidad (*índices biocenóticos*). De esta manera el perfil ecológico traduce cuantitativamente el tipo de relación existente entre la respuesta ecológica y la variable ambiental.

La construcción de estos perfiles mediante análisis frecuencial permite seleccionar las especies indicadoras (Lopez et al., 1997), sensibles o resistentes al vertido de las granjas. Además, al aplicar tests de significación estadística bajo la hipótesis de la equidistribución (ej. Chi cuadrado) se mide el grado de ligazón existente entre la variable respuesta y cada clase preseleccionada de una variable explicativa (Figura 9). Paralelamente se pueden utilizar técnicas de regresión múltiple como una ayuda en la selección de las variables explicativas que forman las ecuaciones que mejor estiman las variables respuesta. Esta información simplifica la futura vigilancia de los escenarios de estudio o de otros similares, al centrarse solamente en unas pocas especies seleccionadas *ad hoc* como indicadores y definir los valores límite de las características físico-químicas clave que serán utilizados como indicadores del impacto ambiental. Carballeira et al., (2011a) a partir de muestras de sedimentos marinos

procedentes de zonas con diferente nivel de afectación, seleccionaron las variables geoquímicas y determinaron los umbrales indicadores del impacto ambiental generado por jaulas instaladas en mar abierto.

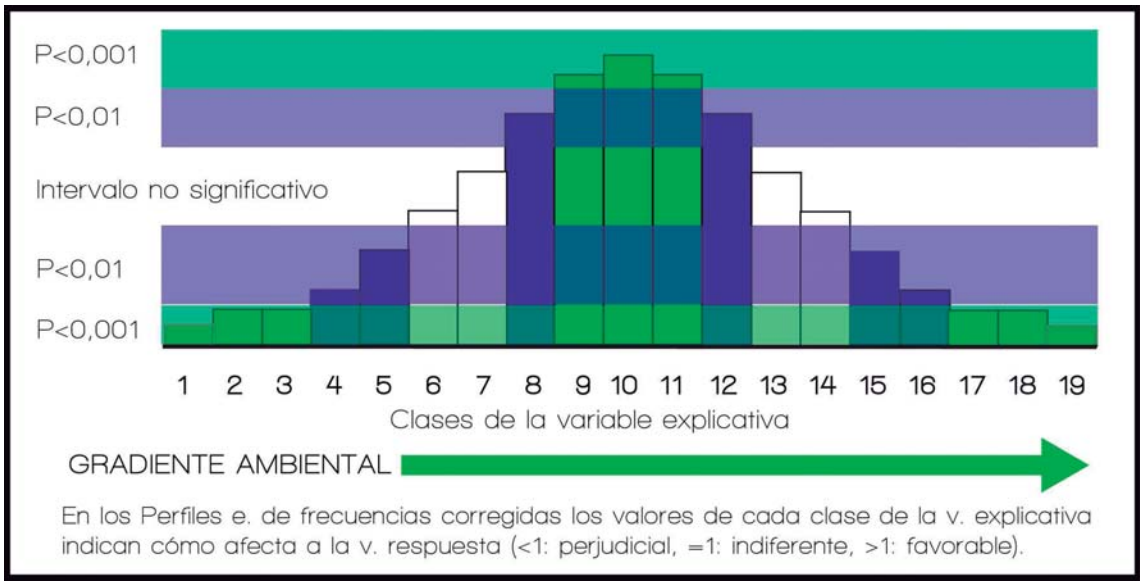


Figura 9. Perfil Ecológico: conjunto de frecuencias corregidas de una respuesta biológica ordenadas por clases de un gradiente ambiental.

2.1.3.5. Biomarcadores

Los biomarcadores se definen como cambios biológicos a nivel bioquímico (por ejemplo, inducción o inhibición de actividades enzimáticas, peroxidación lipídica o daño de ADN), fisiológico (por ejemplo, alteraciones de la reproducción o del crecimiento) o conductual (por ejemplo, alteración de la capacidad natatoria en peces) como consecuencia de la toxicidad de compuestos xenobióticos (Ramos-Gómez et al., 2011). Los biomarcadores se clasifican principalmente en dos grupos: de exposición y de efecto (Galloway et al., 2004). Los biomarcadores de exposición sirven para identificar posibles sustancias a las que los organismos están expuestos. En esta categoría entra, por ejemplo, la actividad de enzimas involucradas en la biotransformación/detoxificación de xenobióticos o aquellas que constituyen el sistema de defensa antioxidante. Los biomarcadores de efecto miden daños que dichas sustancias producen, tales como mutagénesis, genotoxicidad o disrupción endocrina.

El compuesto xenobiótico, una vez dentro del organismo, es sometido en el hepatopáncreas a reacciones de biotransformación que cambian esta molécula de liposoluble a hidrosoluble para que éstas sean fácilmente excretables. Éstas reacciones están catalizadas por enzimas de biotransformación (*biomarcadores de exposición*) de la fase I (alteración no sintética; oxidación, reducción o hidrólisis) y la fase II (conjugación) generando en dicha actividad radicales libres (especies reactivas del oxígeno, ROS) que provocan peroxidación lipídica (LPO), daño de ADN, y daño celular e histológico (*biomarcadores de efecto*) (Van der Oost et al., 2003).

2.1.3.5.1. Biomarcadores moleculares

Los biomarcadores a nivel bioquímico presentan una ventaja fundamental respecto a los cambios en niveles superiores ya que actúan como sistemas de alerta temprana al indicar la presencia de sustancias tóxicas antes de que éstas produzcan daños irreversibles (fisiológicos y conductuales).

Las enzimas del citocromo P450 (CYP) están implicados en la biotransformación de la mayoría de los xenobióticos orgánicos hechos por el hombre. Estas enzimas oxidan, reducen, hidrolizan y conjugan los xenobióticos para facilitar su excreción (Van der Oost et al., 2003). De lo contrario, los xenobióticos podrían interactuar con los constituyentes celulares, alterando su estructura y función, y generando especies reactivas de oxígeno (Reactive Oxygen Species, ROS). En concreto, las enzimas del CYP son conocidas por ser inducidas por compuestos co-planares tales como los bifenilos policlorados, dioxinas, furanos policlorados e hidrocarburos aromáticos policíclicos, que se unen al receptor de aril hidrocarburos (AhR) (Abrahamson et al., 2007; Peters and Livingstone, 2001). También se ha observado una inducción de estas enzimas (e.g. etoxiresorufin O-deetilasa, EROD, y dibenzilfluoresceína, DBF) por la presencia de fármacos (Thibaut and Porte, 2008).

Las ROS pueden resultar no sólo de las interacciones entre los xenobióticos y moléculas endógenas, sino también de la biotransformación de xenobióticos y del metabolismo aeróbico celular. La proliferación de ROS

representa un serio peligro para los organismos vivos, ya que puede oxidar lípidos, proteínas y ADN, alterando su función normal. Sin embargo, con el fin de mantener la salud de organismos las ROS pueden ser neutralizadas por los mecanismos de defensa antioxidante, que comprende enzimas antioxidantes y sistemas no enzimáticos de eliminación de ROS (Van der Oost et al., 2003).

El estrés ambiental causado por la contaminación química, puede provocar un desequilibrio entre la producción de ROS y su eliminación, dando lugar a estrés oxidativo y, en consecuencia, a daño oxidativo. Existe una amplia variedad de contaminantes que están implicados en la aparición de estrés oxidativo; metales, hidrocarburos aromáticos policíclicos, organoclorados, organofosforados y pesticidas, bifenilos policlorados, dioxinas,… (Valavanidis et al., 2006). Por otra parte, estos contaminantes también provocan cambios en el sistema de defensa antioxidante. Por esta razón, los parámetros de estrés antioxidante, incluyendo las actividades enzimáticas y los primeros daños oxidativos, se pueden utilizar para evaluar los efectos perjudiciales de la contaminación sobre la salud de los organismos.

Entre las enzimas más comúnmente usadas en trabajos de contaminación están: la enzima de biotransformación de la fase II, Glutation S-Transferasa (GST), las actividades de la enzimas antioxidantes Glutatión Reductasa (GR) y Glutatión Peroxidasa (GPX), y los efectos oxidativos de la peroxidación lipídica (LPO) y los daños en la cadena de ADN.

2.1.3.5.2. Daño histológico

Los estudios semicuantitativos de alteraciones en los tejidos de bivalvos se han recomendado y utilizado como biomarcadores de efecto para la monitorización de la contaminación marina. Estas alteraciones son sensibles a una amplia gama de contaminantes (Au, 2004), lo que se indica a través del estado de los tejidos diana, proporcionando una visión general de los daños recibidos por los moluscos. El análisis de las alteraciones histopatológicas en los tejidos de los bivalvos nativos (Aarab et al., 2008; Wedderburn et al., 2000) y trasplantados (Morales-Caselles et al., 2008; Nasci

et al., 1999) son de creciente interés en la vigilancia de la contaminación marina debido a su sensibilidad, disponibilidad y relevancia comercial.
Estas técnicas pueden usarse en bioensayos de laboratorio, en bioensayos con organismos trasplantados expuestos a condiciones naturales o con organismos nativos. En este sentido, *Mytilus galloprovincialis* por su amplia distribución y abundancia natural en las costas expuestas a mar abierto puede ser el candidato ideal en Galicia.

2.2. Software informático empleado

A continuación se muestran las distintas aplicaciones informáticas empleadas durante la realización de esta tesis:

2.2.1. Análisis y representación de datos

La aplicación de software informático y componentes lógicos facilitó realizar el análisis estadístico de los datos así como la representación de éstos con calidad suficiente para ser publicados en revistas de divulgación científica.

- *Análisis estadístico*
  - Statistical Package for the Social Sciences (SPSS Statistics, versión 17.0)
  - Microsoft Office Excel 2010
  - R statistical software (versión 2.11.1)

- *Representación de datos*
  - Adobe Ilustrator CS6
  - SigmaPlot (versión 11.0)

2.2.2. Otras aplicaciones informáticas

- Microsoft Office Word 2010 (Procesador de texto)

- Microsoft Office Powerpoint 2010 (Procesador de presentaciones)
- Endnote X4 (Gestor de referencias bibliográficas)
- Foxit pdf Reader (Lector de archivos .pdf)
- Foxit pdf Editor (Editor de archivos .pdf)
- Google Sketchup pro 8 (Diseño 3D)
- Vector Magic Desktop Edition (v1.15)

Artículos del capítulo 2 (ANEXO I)

- *Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas.* **Journal of Environmental Monitoring**.

EVALUACIÓN DE LA EXPOSICIÓN A EFLUENTES PROCEDENTES DE GRANJAS MARINAS INTENSIVAS INSTALADAS EN TIERRA

3.1. Toxicidad potencial

Augas de Galicia, el organismo encargado de la vigilancia ambiental de las granjas de estudio proporcionó datos físico-químicos del agua de entrada y de salida de 18 granjas gallegas pertenecientes al período 2000-2008. Tras realizar las medias de los parámetros de acuerdo al agua de entrada y al vertido se observó un descenso medio del pH de 3 décimas respecto al agua de entrada, así como un incremento de los sólidos en suspensión, COT (*Carbono Orgánico Total*), fosfatos y de las distintas formas del N (nitritos, nitratos y amonio). Además, se midieron los mismos parámetros físico-químicos de los vertidos muestreados en las 8 granjas de estudio y se observaron valores similares o de peor calidad que la media de los efluentes (Carballeira et al., 2012a). Las medidas físico-químicas del vertido representan un análisis de *screening* que ha de ser empleado dentro del PVA debido a su bajo coste y a la cantidad de información proporcionada.
También se realizaron análisis de los vertidos con el fin de detectar pesticidas, antibióticos y metales comunes en acuicultura, pero todos ellos se encontraron por debajo de los límites de detección. Además, todas las concentraciones de metales estaban por debajo de la concentración media de fondo determinada para aguas meso-polihalinas (Tueros et al., 2008). El elevado coste de este tipo de análisis y el desconocimiento de las sustancias, que pueden ser usadas de manera singular, limita la utilidad de estas medidas para ser incluidas dentro del PVA.

3.2. Área de influencia

3.2.1. Biomonitores seleccionados

La presencia de contaminantes en el medio fue evaluada en términos de bioacumulación en organismos nativos y trasplantados. Para ello se tuvieron que seleccionar especies abundantes, sésiles y de distintos niveles tróficos, que se utilizarían como biomonitores: macroalgas nativas y trasplantadas, anémonas y mejillones nativos (Figura 10).

Se recolectaron varias especies de macroalgas nativas debido a la no existencia de una única especie común a todos los escenarios y puntos de muestreo. Las especies seleccionadas pertenecen al género *Fucus* sp. (*F. vesiculosus, F.spiralis* y *F. serratus*), algas marrones ampliamente estudiadas, cosmopolitas y habituales en las costas gallegas, y el alga verde *Codium tomentosum,* abundante en las zonas próximas a los vertidos ricos en nitrógeno.

Paralelamente se realizaron transplantes a modo de gradiente de la macroalga *Laminaria saccharina* L., importante por sus propiedades medicinales y el respectivo interés comercial.

Se constató *de visu* una elevada presencia de anémonas en las zonas afectadas por el vertido por lo que se decidió seleccionar la especie *Anemonia sulcata L.* como biomonitor.

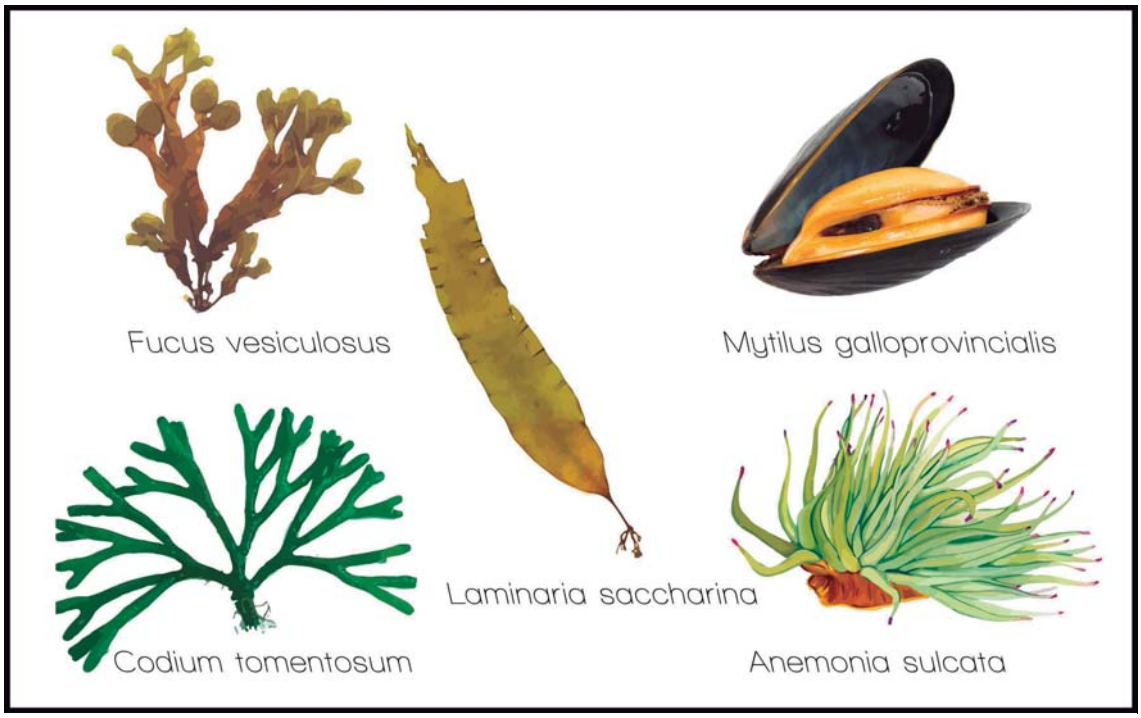


Figura 10. Representación gráfica de los distintos biomonitores empleados en esta tesis.

Otro organismo común en las costas gallegas, ampliamente estudiado por su importancia comercial, es el bivalvo *Mytilus galloprovincialis* Lamarck (1819). Este molusco no sólo fue seleccionado para comprobar la bioacumulación de microcontaminantes sino también para el estudio de la integridad ecológica a través del análisis de biomarcadores de exposición y de efecto.

3.2.2. Determinación de marcadores de exposición

3.2.2.1. Metales y metaloides

Algunos metales, como Cd, Cr, Cu, Pb y Zn, han sido asociados a la actividad piscícola por ser constituyentes de desinfectantes y de productos anti-fouling y porque también están presentes en la dieta de los peces (Dean et al., 2007). Sin embargo, se realizó un análisis cualitativo de numerosos metales y metaloides (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hg, In, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Pd, Pt, Rb, Sb, Sc, Se, Si, Sn, Sr, Te, Ti, V, W, Y, Zn y Zr) con el fin de detectar la posible bioacumulación de otros elementos no recogidos en la bibliografía. Se encontraron pequeños incrementos en la concentración de determinados elementos (Al, Cu, Hg, Ni y Pb) bioacumulados por *Laminaria saccharina* y *Anemonia sulcata* recolectadas en zonas cercanas al vertido, sin embargo, no se encontraron diferencias significativas entre los distintos puntos de muestreo dentro de cada gradiente para el resto de biomonitores (Rey-Asensio et al., 2010).

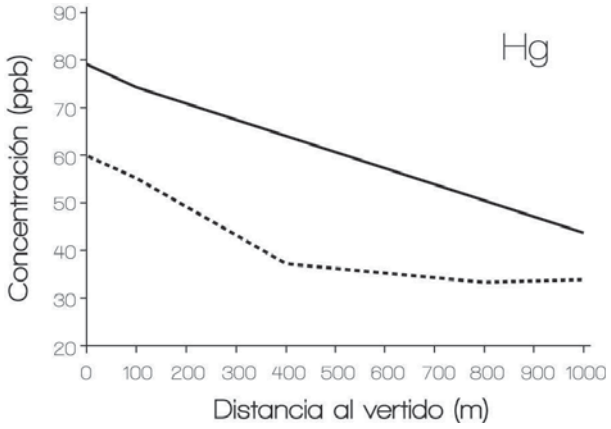


Figura 11. Concentración corporal de Hg (ppb) en *Anemone sulcata* recolectada en el área de influencia de dos piscifactorías marinas instaladas en tierra (LBMFF) (Granja II y IV).

Destacó la presencia de Hg en anémona. El origen del Hg podría estar relacionado con el alimento (harina de pescado) suministrado. Tras obtener

los resultados del análisis cualitativo y debido a la elevada toxicidad del Hg se realizó un análisis cuantitativo de éste en todos los biomonitores seleccionados. Se observó un gradiente claro de disminución de Hg en todos ellos pero especialmente en *L. saccharina y A. sulcata* (Figura 11).

3.2.2.2. Antibióticos y pesticidas

Se realizó un sondeo de la presencia de antibióticos y pesticidas en las muestras de *L. saccharina* trasplantada en dos estaciones de muestreo, situadas a 50 m y 800 m del foco, de dos granjas (Granja I y IV).

Se analizaron los antibióticos autorizados más utilizados en el cultivo de peces planos: amoxicilina, oxitetraciclina, ácido oxolínico, flumequina, sulfadiazina, ampicilina y estreptomicina. Aunque se encontraron diferencias en la bioacumulación de antibióticos entre las dos estaciones, las concentraciones de estos antibióticos se encontraron, en todos los casos, por debajo de los límites de seguridad establecidos para la alimentación humana. Respecto a los pesticidas, al tener desconocimiento de cuales podían estar presentes, se realizó un screening cualitativo. Algunos de los pesticidas, tales como prometrina, prometón y clorotalonil fueron detectados en pequeñas concentraciones en las algas trasplantadas (Rey-Asensio et al., 2010).

Los escasos resultados obtenidos y su elevado coste descartan la utilidad del análisis de microcontaminantes en el medio o en organismos (bioacumulación) como herramienta para obtener información sobre la extensión y el grado del impacto potencial del vertido, sobre todo si lo comparamos con la información suministrada por la señal isotópica $\delta^{15}N$ en macroalgas.

3.2.2.3. Señal isotópica $\delta^{15}N$

La medida de la relación isotópica $\delta^{15}N$ reveló ser un excelente marcador de exposición en todos los biomonitores analizados, mostrando un evidente gradiente de contaminación (Rey-Asensio et al., 2010). Por este motivo, se decidió estudiar los valores de fondo, la variabilidad estacional y temporal y

las diferencias interespecíficas en la bioacumulación de la señal isotópica de $\delta^{15}N$.

La $\delta^{15}N$ presentó escasas diferencias interespecíficas entre las macroalgas nativas. Por ello, se calcularon únicamente los valores de fondo regionales para la macroalga más común en Galicia (*Fucus vesiculosus)* y para facilitar la interpretación e incorporación de datos procedentes de un banco de muestras ambientales (BEAG, Banco de Especimenes Ambientales de Galicia) (Figura 12).

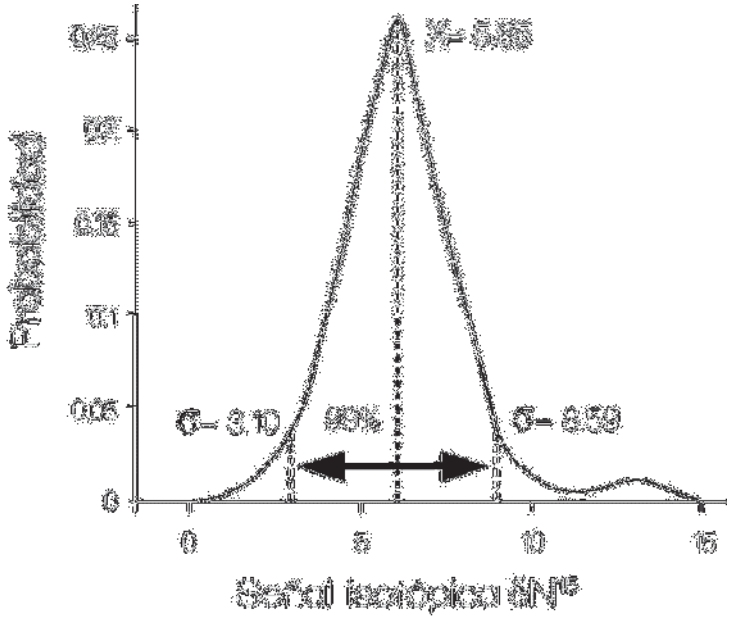


Figura 12. Rango de referencia regional de la relación isotópica $\delta^{15}N$ en la especie piloto *Fucus vesiculosus.*

También se observó una baja variabilidad estacional de la señal $\delta^{15}N$ en macroalgas nativas (Carballeira et al., 2012e). Aun así, como estas variaciones en la señal están directamente relacionadas con la carga contaminante, se recomienda su determinación en la época de mayor producción, finales de verano y principios de otoño (Julio-Octubre).

Por otro lado, hubo una disminución en los valores de $\delta^{15}N$ con el tiempo, probablemente debido a la eliminación del $\delta^{15}N$ externamente adherido (partículas), la variabilidad de las condiciones ambientales o un aumento en la tasa de producción piscícola.

Se observaron valores muy bajos de la señal $\delta^{15}N$ en el punto más cercano al foco de contaminación, probablemente debido a la acidificación del

medio, causada por el vertido y, que dificulta la absorción del nitrógeno amoniacal. Además, la inestabilidad interanual de la señal $\delta^{15}N$ obliga a estandarizar el tiempo de exposición de los biomonitores. Sin embargo, los mayores valores de $\delta^{15}N$ de los sitios impactados claramente demuestran la influencia de las granjas de peces en el medio marino y su utilidad en los PVA de estas granjas.

El análisis de la señal $\delta^{15}N$ fue también incluido en los bioensayos pertenecientes a la integridad ecológica (crecimiento de discos de *Ulva* sp., fitoplancton y colonización de sustratos artificiales) debido a su importancia como descriptor del riesgo de eutrofización y por la posibilidad de correlacionar dicho parámetro con los efectos causados en el medio. En todos los casos, la señal $\delta^{15}N$ mostró una clara relación exposición-efecto.

Artículos del capítulo 3 (ANEXO II)

Evaluación de la exposición

- *Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioacumulación de microcontaminantes.* **Foro dos recursos mariños e da acuicultura das rías Galegas.**
- *$\delta^{15}N$ values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms.* **Journal of applied Phycology.**
- *Interannual changes in $\delta^{15}N$ values in Fucus vesiculosus L.*

EVALUACIÓN DE LA ecoTOXICIDAD ESPECÍFICA GENERADA POR LOS EFLUENTES PROCEDENTES DE GRANJAS MARINAS INTENSIVAS INSTALADAS EN TIERRA

4.1. Selección de organismos y criterios

Para una correcta evaluación de los efectos tóxicos y tróficos es necesario seleccionar de forma adecuada los distintos organismos que serán expuestos al vertido, las condiciones en las que se realizarán los bioensayos y los criterios de toxicidad (*endpoints*) más significativos y precisos.

Se preseleccionó una batería de bioensayos con organismos pertenecientes a distintos niveles tróficos (Peters et al., 2002): un productor primario (microalga), un descomponedor (bacteria) y un consumidor (erizo de mar). Para ello, los criterios de priorización fueron: selección de especies nativas, facilidad de cultivo y mantenimiento, sensibilidad a los vertidos de las granjas y que la metodología estuviese lo más estandarizada posible para permitir comparar nuestros resultados con aquellos obtenidos en otros estudios de toxicidad.

4.1.1. Bioensayo con bacteria

Se escogió el bioensayo estandarizado de Microtox®, que emplea la bacteria marina *Vibrio fischeri,* para la evaluación toxicológica del principal producto de la excreción de peces (amonio), y de antibióticos (amoxicilina, ampicilina, flumequina, oxitetraciclina, estreptomicina y sulfadiazina) y desinfectantes (NaClO y CH_2O) comúnmente usados en acuicultura.

Este bioensayo se llevó a cabo con distintos tiempos de exposición (endpoints a los 15, 30 y 60 minutos) y medios de cultivo (salinidad al 2 y al 3.7%) con el fin de seleccionar los criterios más sensibles a los contaminantes presentes en el vertido (Viana et al., In press). Además, la metodología de este bioensayo se adaptó para su miniaturización de manera que las

lecturas fueran realizadas directamente sobre una microplaca, de forma más rápida y económica.

El test de microtox realizado con agua de mar al 2% (método estándar) y 60 minutos de exposición resultó ser el más sensible (Viana et al., In press). Sin embargo, estas condiciones no reflejan la toxicidad real de los contaminantes ya que en muchos casos la toxicidad disminuye con el aumento de salinidad o aumenta con el tiempo de exposición (*toxicidad retardada*). Para la correcta evaluación de los vertidos de estas granjas sería necesario estandarizar el método usando mayores salinidades (siempre y cuando este incremento no perjudique directamente a la bacteria) y tiempos de exposición (una mayor exposición puede aumentar la capacidad de detección de los tóxicos).

En todo caso, este bioensayo resultó ser suficientemente sensible al contenido de los vertidos de piscifactorías marinas en tierra y, por lo tanto, es un candidato claro a ser incluido en la batería mínima de bioensayos de laboratorio para la evaluación la toxicidad de los vertidos de estudio.

4.1.2. Bioensayo con microalga

Al igual que el bioensayo bacteriano anterior, el bioensayo de microalgas fue miniaturizado y empleado para la evaluación toxicológica de las mismas sustancias y vertidos. En este caso el bioensayo se realizó de forma paralela con dos especies de microalgas: *Phaeodactylum tricornutum* (AROSA) e *Isochrysis galbana,* con la finalidad de escoger la especie más sensible que compondría la batería de bioensayos (DeOrte et al., *en prensa*).

Aunque los resultados obtenidos muestran la sensibilidad selectiva de las microalgas (depende del tipo de compuesto), por lo general, el bioensayo con *P. tricornutum* resultó ser más sensible y de mejor replicabilidad. Asimismo, su relevancia se incrementa en este estudio al tratarse de una microalga obtenida de las costas gallegas (Fabregas et al., 1984) que no se verá afectada por las condiciones ambientales naturales de la zona de estudio y evitará la aparición de ruidos innecesarios en el bioensayo.

4.1.3. Bioensayo con erizo de mar

En este bioensayo se utilizaron las fases tempranas, embrión y larva, del erizo de mar por su mayor sensibilidad en comparación con las fases adultas y organismos de niveles tróficos superiores (Carballeira et al., 2011c). En estos bioensayos la medida de toxicidad de cada muestra se contabilizó como el porcentaje de huevos no fecundados (test de fertilización) o larvas deformes (test de desarrollo larvario).

En Europa, por lo general, los bioensayos estandarizados con erizo de mar emplean la especie *Paracentrotus lividus* Lamarck (1816). La realización del bioensayo con larvas de erizo depende de la disposición de adultos maduros o gametos fértiles y las técnicas de criogenización de los gametos de erizo de mar todavía no permiten conservar la fertilidad de los huevos durante más de unos días (Paredes and Bellas, 2009).

Existe una especie de erizo de mar, conocido como erizo negro (*Arbacia lixula* Linnaeus, 1758) que comparte hábitat, presenta mayor distribución y mayor período de fertilidad que *P. lividus* (Carballeira et al., 2011c) (Figura 13). Algunas referencias verifican incluso la fertilidad continua de la población a lo largo de todo el año. De esta manera, la realización del bioensayo de toxicidad empleado con esta especie eliminaría la principal limitación operativa del bioensayo realizado con *P. lividus*.

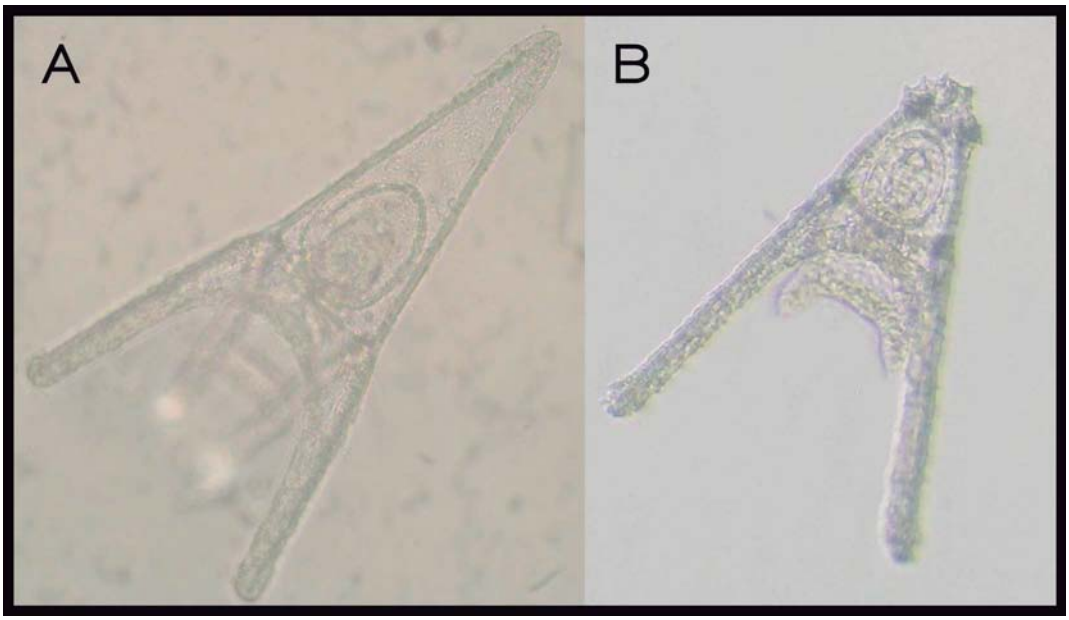


Figura 13. Estado de larva (*pluteus*) del erizo común (**A**, *Paracentrotus lividus*) y del erizo negro (**B**, *Arbacia lixula*).

Se estudió la respuesta a la salinidad de dos tipos de bioensayos con erizo de mar, el test de fertilización y el test de desarrollo larvario, usando ambas especies. Las larvas de erizo negro mostraron un mayor rango óptimo de salinidad, mientras que hubo problemas para identificar de manera visual la membrana de fertilización de los huevos de esta especie (Carballeira et al., 2011c). De este modo, la aplicación del test de fertilización con *A. lixula* necesitará profundizar en el estudio de la técnicas de detección de la membrana para una correcta medida de la toxicidad.

Posteriormente se expusieron las larvas de ambas especies a disoluciones de las mismas sustancias que se emplearon con bacteria y microalgas (amonio, antibióticos y desinfectantes) para ver que especie era la potencialmente más sensible a los vertidos de las granjas (Carballeira et al., 2012b). Prácticamente no hubo diferencias entre las sensibilidades de ambas especies y también se observó, en ambas especies, que elevadas concentraciones de algunos antibióticos mejoraban las condiciones de desarrollo de las larvas de erizo, llegando incluso a mostrar menores porcentajes de larvas deformes que los controles.

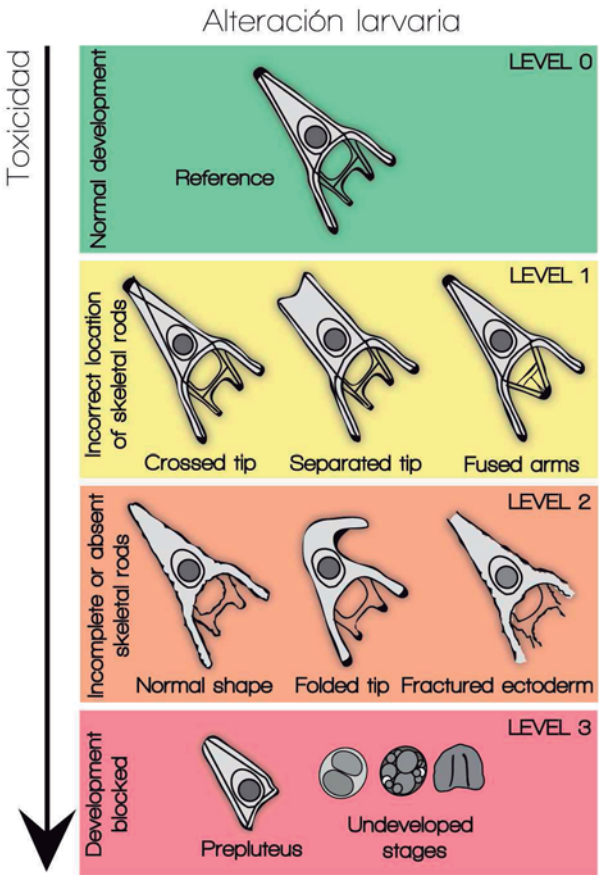


Figura 14. Criterios de toxicidad establecidos en función de las deformidades observadas en larvas de erizo expuestas a los vertidos de las piscifactorías marinas instaladas en tierra (LBMFF).

Durante la realización de los bioensayos con *P. lividus* y vertidos procedentes de granjas se observó que los vertidos y el amonio causaban deformaciones características en el esqueleto de las larvas de erizo (Carballeira et al., 2012c). En algunos casos el esqueleto desaparecía por

completo sin alterar la forma singular de las larvas. Aunque ya se han hecho algunos estudios sobre este tipo de deformaciones, éstas no están incluidas en ningún protocolo y el criterio comúnmente usado es el de larva normal y anormal según conserve los brazos y la forma característica de la larva.

Las distintas deformaciones esqueléticas encontradas en las larvas de erizo de mar fueron clasificadas y caracterizadas lo cual permitió establecer distintos niveles de toxicidad dependiendo de la severidad del daño (Carballeira et al., 2012c) (Figura 14). Esto abre nuevas perspectivas, así futuros bioensayos de laboratorio con larvas de *P. lividus* frente a mezclas complejas de los contaminantes estudiados u otros podrían suministrar modelos que correlacionen la composición del vertido con deformidades singulares facilitando así la monitorización ambiental.

4.2. Evaluación de la ecotoxicidad de los vertidos

Para la evaluación de los efectos causados por los vertidos se recogieron muestras de 8 granjas durante las horas en las que los peces se encuentran metabólicamente más activos (8am-8pm) y durante los meses de máxima producción, con el fin de reflejar el máximo impacto potencial medio diario de estas granjas.

La batería formada por los bioensayos de laboratorio anteriores se utilizó para integrar los efectos tóxicos provocados por los vertidos en cada organismo y expresar dicha toxicidad como un valor numérico (*índice PEEP,* Potential Ecotoxic Effects Probe) que está directamente relacionado con la cantidad de residuos liberados al medio (Carballeira et al., 2012a).

Dentro de este estudio se observaron las sensibilidades de los distintos bioensayos y especies hacia los vertidos y se seleccionaron: 1/ las especies y métodos más sensibles (*V. fischeri* de 30 min, la tasa de crecimiento poblacional de *I. galbana* y deformaciones larvarias de *A. lixula*), 2/ los vertidos más tóxicos, 3/ la dilución que mejor explica la variabilidad entre granjas (1:4) y 4/ los parámetros ecotoxicológicos más apropiados (EC_{20}) para ser usados en los planes de vigilancia ambiental (Carballeira et al., 2012a).

Por lo general, los vertidos mostraron una baja toxicidad que, en algunos bioensayos con microalgas, llegó a estimular el desarrollo de estos organismos al ser ésta menor que los efectos tróficos generados por los nutrientes del vertido. Aun así, el empleo conjunto de las tres especies test resultó ser una herramienta apropiada para la evaluación de los efectos tóxicos y tróficos de los vertidos. Se diseñó una representación gráfica de los resultados que permite establecer e interpretar fácilmente y de manera integrada la toxicidad de cada vertido (Figura 15).

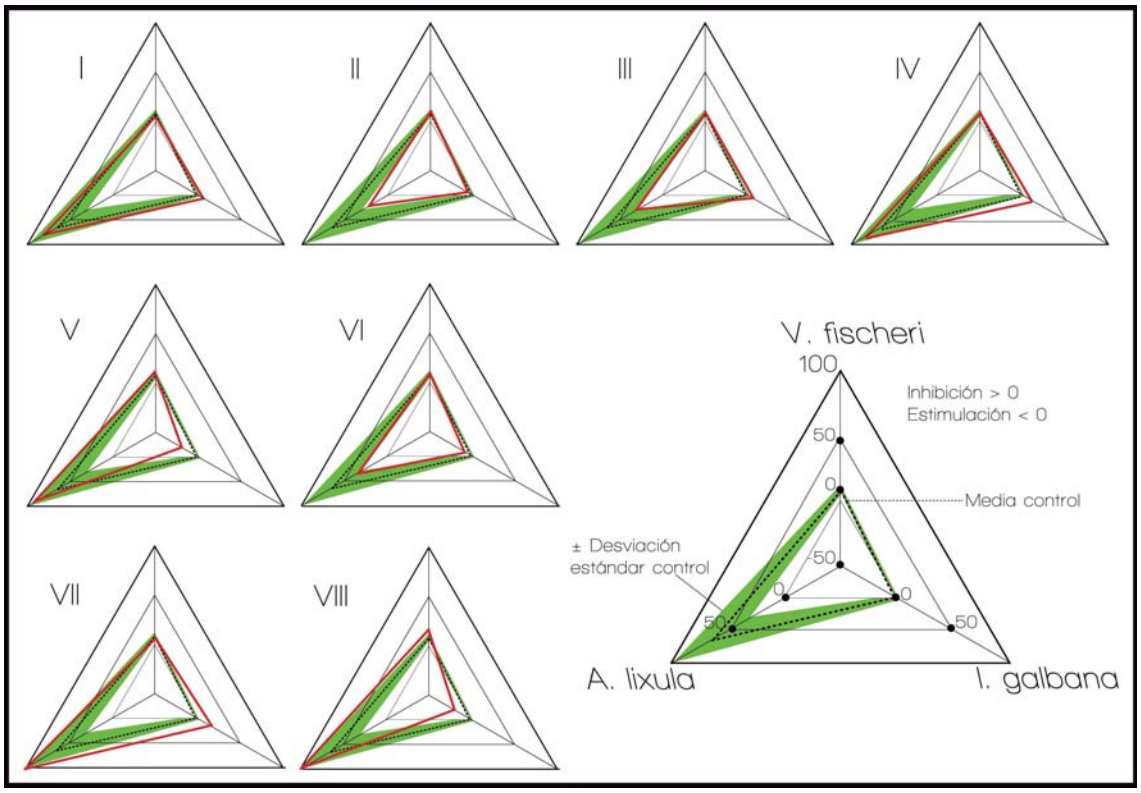


Figura 15. Perfiles ecotoxicológicos de los vertidos –para la dilución 1:4 (v/v)- procedentes de ocho piscifactorías marinas instaladas en tierra (LBMFF).

Artículos del capítulo 4 (ANEXO III)

Selección de organismos y criterios

- *Influence of salinity on fertilization and larval development toxicity tests with two species of sea urchin.* **Marine Environmental Research.**
- *Identification of specific malformations of sea urchin larvae for toxicity*

assessment: Application to marine pisciculture effluents. **Marine Environmental Research.**

- *Assessing the toxicity of chemical compounds associated with marine land-based fish farms: The use of mini-scale microalgal toxicity tests.* **Chemistry and Ecology**.
- *Evaluation of the toxicity of commonly used biocides in land based marine fish farms using bioluminescence test miniaturized Vibrio fischeri.*
- *Assessing the toxicity of chemical compounds associated with land-based marine fish farms: the sea urchin embryo bioassay with Paracentrotus lividus and Arbacia lixula.* **Archives of Environmental Contamination and Toxicology.**

Evaluación de los efectos

- *Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms.* **Ecotoxicology and Environmental Safety.**

EVALUACIÓN DE LA ALTERACIÓN DE LA INTEGRIDAD ECOLÓGICA DE LAS ZONAS AFECTADAS POR LOS VERTIDOS DE LAS GRANJAS MARINAS INTENSIVAS INSTALADAS EN TIERRA

La integridad de un ecosistema se consigue cuando todos sus componentes, bióticos y abióticos, y las interacciones entre ellos se encuentran inalterados, para ello no sólo la composición y estructura han de estar intactas sino también las funciones y los procesos que los mantienen (Wipond and Dearden, 1998). Un sistema ecológico mantendrá la integridad cuando todos sus sistemas naturales estén funcionando correctamente. Si las interacciones entre los componentes de los ecosistemas se interrumpen, el sistema pierde su integridad. De esta forma, cualquier alteración ocasionada a las poblaciones del área de influencia de una perturbación servirá para expresar la alteración de la integridad ecológica.

Tradicionalmente la medida de la alteración de la integridad ecológica se basa en la elaboración de índices bióticos con organismos macro o microscópicos. En general este tipo de aproximaciones requieren un laborioso trabajo, poco operativo a nivel local, impreciso y a veces destructivo para el medio.

Se escogieron los bioensayos *in situ* realizados a nivel específico con trasplantes de organismos y a nivel pluriespecífico los bioensayos de colonización de sustratos artificiales (fouling) y los bioensayos realizados con trasplantes de la comunidad fitoplanctónica por la facilidad y precisión de sus medidas al regularizar la exposición, y por ser respetuosos con el medio. Por otro lado, se muestreó en el área de influencia de las granjas *Mytilus galloprovincialis*, el molusco más abundante y representativo de las costas gallegas, para analizar una batería de biomarcadores de exposición y de efecto (moleculares e histológicos) a los vertidos.

La integración de los datos suministrados por el conjunto de bioensayos realizados *in situ* desde el nivel molecular al de comunidad nos proporcionará una valiosa información del estado "real" en el que se

encuentran las poblaciones afectadas por el vertido y de la extensión del área impactada.

5.1. Bioensayos *in situ*

5.1.1. Bioensayo con discos de macroalgas

El objetivo de este bioensayo era comprobar el efecto *in situ* de los vertidos sobre la macroalga oportunista *Ulva* spp. como representante de los productores primarios bentónicos. Para realizar los bioensayos se obtuvieron discos de *Ulva* sp. de 17 mm de diámetro y se dispusieron 25 en cada cámara de incubación (Figura 18). La cámara de incubación consiste en un cilindro acrílico transparente de 10 cm de diámetro y 20 cm de largo que se cierra por los extremos con red de nylon de 1,5 mm de malla (Figura 7). En cada estación de muestreo se dispusieron 4 cámaras a 1,5 m bajo la superficie durante 6 días. El experimento se realizó en dos escenarios estableciendo cinco estaciones de muestreo por escenario.

Tras la extracción de los discos de la fronde principal se produce la activación de la esporulación a partir de los bordes en un porcentaje alto de los discos, los cuales sufren una decoloración pigmentaria y a continuación pueden llegar a deshacerse y desaparecer, lo que se conoce como "*ghost tissue*" (Salomonsen et al., 1999). Este fenómeno reduce el número de discos al final del período de exposición y la fiabilidad del bioensayo. Mediante experimentación en laboratorio y campo comprobamos que se puede evitar la "desaparición" de los discos, por "cicatrización" con una disolución NaClO al 0,05 % durante 60 segundos (Viana et al., 2009).

Al final del período de exposición se estudia el efecto de los vertidos sobre los discos respecto a: la concentración de N, la señal isotópica $\delta^{15}N$, la concentración de clorofilas *a* (Chl a) y *b* (Chl b), los índices pigmentarios (Chl a/Chl b, D_{665}/D_{665a}, $D_{430/410}$), la fluorescencia clorofílica (φPSII) y la tasa de crecimiento relativa (Relative Growth Rates, RGR) en función del incremento neto de biomasa.

La concentración de N y de la señal isotópica ($\delta^{15}N$) aumentaban gradual y significativamente al aproximarse al foco contaminante en ambos escenarios, sin embargo, no se observaron diferencias significativas en las concentraciones de clorofilas, índices pigmentarios y fluorescencia clorofílica, lo que indica ausencia de estrés en los discos trasplantados. Hemos de señalar que los discos de ulva presentaban en el tiempo cero (t_0) un valor de $\delta^{15}N$ elevado (8,3±0,6 ‰), de ahí que se encontraran porcentajes negativos al final del periodo de exposición, sobre todo en las estaciones limpias, las más alejadas del foco.

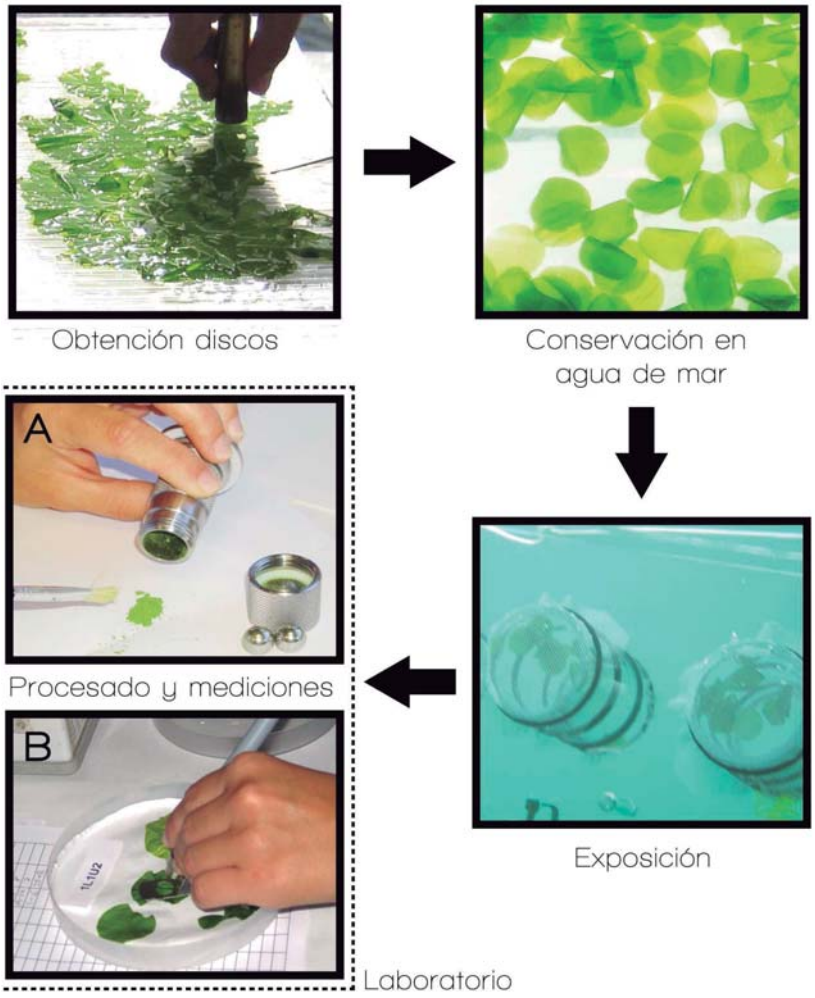


Figura 18. Bioensayo de fertilidad-toxicidad realizado con discos de *Ulva* sp. **A.** Molienda de la muestra para el posterior análisis químico. **B.** Lectura de la fluorescencia clorofílica de los discos.

Los vertidos inhibían de manera significativa el crecimiento (RGR) de los discos de *Ulva* sp. La falta de una respuesta gradual con la distancia al foco y la ausencia de diferencias significativas entre las cinco estaciones de

cada escenario para los parámetros de estrés indican que los efluentes parecen no afectar ni a la acumulación de pigmentos fotosintéticos ni al proceso de la fotosíntesis. A pesar de que existe un evidente gradiente de exposición a nutrientes (% N y $\delta^{15}N$), la biomasa aumenta al alejarse del foco. Al comparar estos resultados con la resistencia de la *Ulva* sp. obtenida con los perfiles ecológicos parece indicar, al igual que en el caso del bioensayo de fitoplancton, un mayor efecto tóxico que trófico en los estaciones más cercanas al punto de vertido.

Ulva sp. es una especie oportunista y resistente al vertido pero su crecimiento neto relativo sólo aumenta a partir de un cierto grado de reducción del efecto tóxico. Los tóxicos se diluyen rápidamente debido a su baja concentración, mientras que los efectos tróficos (nutrientes) se mantienen en una mayor distancia dentro del área de influencia donde los organismos fotosintetizadores se encuentran saturados de nutrientes.

5.1.2. Bioensayo de la comunidad de fitoplancton

El objetivo de este estudio es evaluar la dispersión y el impacto ecológico de los vertidos de las piscifactorías sobre la comunidad fitoplanctónica nativa confinada en bolsas de diálisis (Figura 17). Para la realización de los bioensayos se introdujo en bolsas de diálisis una muestra de fitoplancton de la comunidad nativa existente fuera del área de influencia de las granjas después de ser filtrada a 50µm con el objeto de eliminar el pastoreo del zooplancton herbívoro. Las bolsas de diálisis con la comunidad fitoplanctónica nativa se expusieron en forma de gradiente en el área de influencia de dos granjas. En cada granja se localizaron cinco estaciones de muestreo y en cada estación de muestreo se dispusieron 4 bolsas de diálisis de 700 ml de capacidad. Después de 2 días de exposición se determinó el índice de feofetinización (D_{665}/D_{665a}), la fluorescencia clorofílica (φPSII), el contenido de clorofila *a* y la señal isotópica $\delta^{15}N$ de la comunidad fitoplanctónica incubada (Figura 17). Las diferencias significativas encontradas entre puntos de muestreo consecutivos indican el área de influencia que tienen los vertidos en los parámetros analizados.

Las medidas de la clorofila en masas de agua son comúnmente utilizadas por estar relacionadas con la biomasa y la productividad primaria

fitoplanctónica pero presentan una elevada variación espacial y temporal, sobretodo en costas con elevado hidrodinamismo. Se han realizado estudios de este tipo, respecto a piscifactorías en mar abierto en los que se observa un incremento de estos parámetros al acercarse a las jaulas debido al efecto eutrofizador de sus residuos. Sin embargo, al aplicar este estudio en las proximidades de granjas intensivas se observa que existe un elevado aporte de nutrientes cerca del foco (señalado por el incremento de la señal $\delta^{15}N$) pero los parámetros indicadores de crecimiento se mantenían en todos los casos por debajo de los valores considerados como blancos (la condición inicial t_0 y el punto más alejado del foco, BS5). Este comportamiento podría ser debido: 1/ la escasa duración del período de incubación, sin embargo, su aumento conlleva problemas de reciclado interno y deterioro de la membrana de diálisis; 2/ que los efectos tóxicos del vertido se superponen a los estímulos tróficos, lo que indicaría la existencia de productos químicos inhibidores en el vertido; 3/ que los cambios en las propiedades físico-químicas del medio (*efecto trasplante*) se superpongan a cualquier otro posible efecto; o 4/ a la combinación de todo los efectos. Los resultados obtenidos con este bioensayo en estas áreas con alto hidrodinamismo resultaron poco satisfactorios y a falta de nuevos estudios se desecharía su inclusión en los PVA.

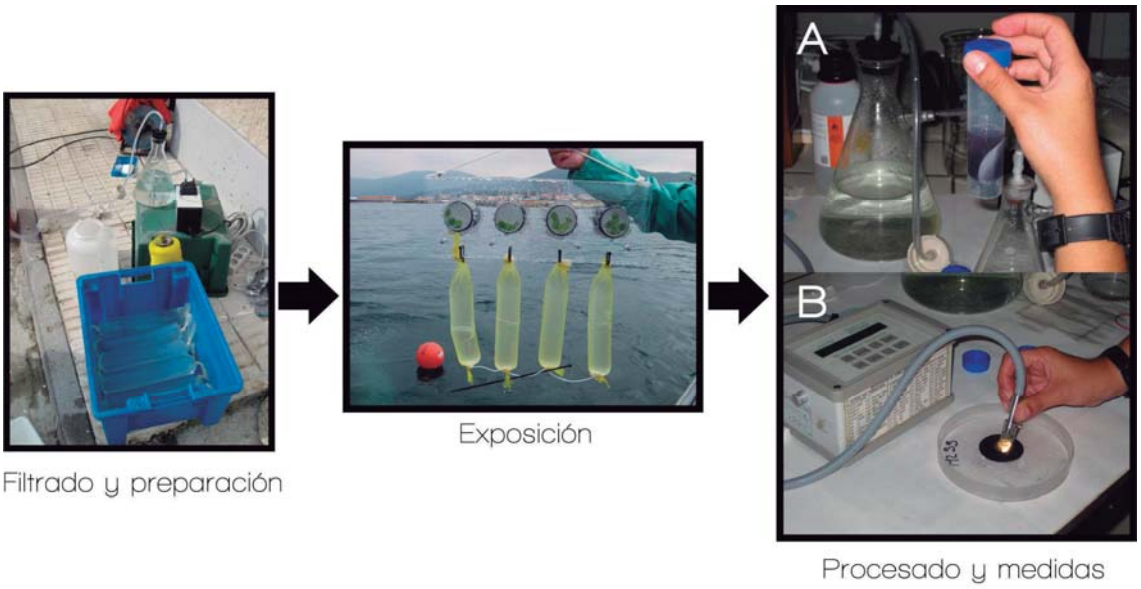


Figura 17. Bioensayo de la comunidad de fitoplancton nativo. **A.** Extracción de los pigmentos del fitoplancton retenido en el filtro. **B.** Lectura de la fluorescencia clorofílica.

5.1.3. Bioensayo de la comunidad colonizadora de sustratos artificiales

Se dispusieron en gradiente estructuras artificiales para su colonización en dos escenarios. El bioensayo tuvo que ser repetido porque el diseño inicial de las superficies artificiales presentaba una serie de inconvenientes, como pérdidas de biomasa por rozamiento con el dispositivo de fijación y efecto de borde (Figura 8). Posteriormente, se utilizaron estructuras esféricas para evitar los inconvenientes citados, además de asegurar que las superficies expuestas reflejarían de manera estable las distintas condiciones de luminosidad (Figura 7). De esta forma se genera un gradiente de iluminación desde el polo del hemisferio superior, el más iluminado, que será colonizado fundamentalmente por organismos autótrofos hasta el polo del hemisferio inferior donde dominarán los consumidores. Esta diversificación del ambiente permitirá matizar (deslindar) mejor las respuestas funcionales y estructurales de la comunidad colonizadora a los vertidos de las granjas.

Entre las respuestas funcionales del fouling de fácil obtención se considera la composición pigmentaria de los productores primarios, la biomasa y la señal isotópica, esta última como medida de la disponibilidad de nitrógeno y como descriptor del grado de exposición.

El grado de alteración de la composición y estructura de la comunidad de fouling instalada sobre los sustratos artificiales puede ser caracterizada mediante la abundancia de especies indicadoras o a través de los valores que toman determinados índices biocenóticos.

La detección de bioindicadores puede realizarse *de visu* cuando en condiciones de campo rutinarias las evidencias son claras y la vigilancia se reduce a unas pocas especies. La búsqueda y caracterización de indicadores clave se basa en múltiples observaciones y en el establecimiento de relaciones numéricas entre el indicador y el objeto a indicar (p.e. un factor fisico-químico, un índice de calidad,…). La base de estas técnicas radica en que las características de las especies indicadoras (abundancia, vigor, etc.) varían a lo largo de gradientes ambientales. Para la estima de la intensidad o grado de asociación entre bioindicadores y gradientes ambientales las técnicas de análisis frecuencial son muy adecuadas. El análisis frecuencial (Lopez et al., 1997) se basa en la

construcción y caracterización de Perfiles Ecológicos (PE). Originalmente un PE es una serie ordenada de frecuencias de las presencias de una especie en las clases de una variable (i.e. pH; salinidad, concentración de un contaminante) o de un descriptor (i.e. profundidad del agua; distancia al foco de emisión). Pero un PE se puede construir para cualquier tipo de respuesta biológica, desde un biomarcador molecular hasta un parámetro macroscópico como la diversidad específica de una comunidad.

Para la construcción de los perfiles ecológicos primero se analizan la composición de la comunidad en cada superficie de colonización. La composición de la comunidad puede ser caracterizada a nivel de especie, familia, grupo trófico, etc. A continuación se depura el inventario eliminando los caracteres raros (presencia menor al 5%). Posteriormente se calcula la abundancia de cada carácter y se ordenan los datos, en clases de distancia al foco. Se calculan los valores medios y máximos de cada clase y se transforman los datos de cada clase en frecuencias corregidas respecto al perfil global, dividiendo el valor de cada clase por la media de los valores de todas las clases del perfil; a continuación el dato obtenido se le aplica el logaritmo para que las respuestas positivas y negativas respecto al valor esperado sean simétricas. El cálculo de la significación estadística de la respuesta (+ o -) de cada clase del perfil se realiza a través de las tablas de contingencia y de la prueba Chi cuadrado (χ^2).

La composición y estructura de la comunidad colonizadora resultado de la exposición al vertido puede ser debida: a la toxicidad del vertido, a su efecto fertilizador o a una combinación de ambos (Carballeira et al., 2012d). La construcción de los perfiles ecológicos de frecuencias corregidas permitió seleccionar los caracteres indicadores de la calidad del vertido (Figura 16). Como ejemplo de especies **bioindicadores resistentes** a los vertidos de las granjas están: *Dermocarpaceae spp, Ulva spp y Anfípodos tubícolas,* y como **bioindicadores sensibles:** *Diatomeas bentónicas, Hincksia granulosa, Rhodothamniella caespitosa.*

El conocimiento del comportamiento de unos pocos bioindicadores y el uso de dispositivos de muestreo localizados en gradiente permitirán agilizar y estandarizar los estudios sobre el grado de alteración de la integridad ecológica de las comunidades expuestas al vertido en función de la distancia al foco emisor.

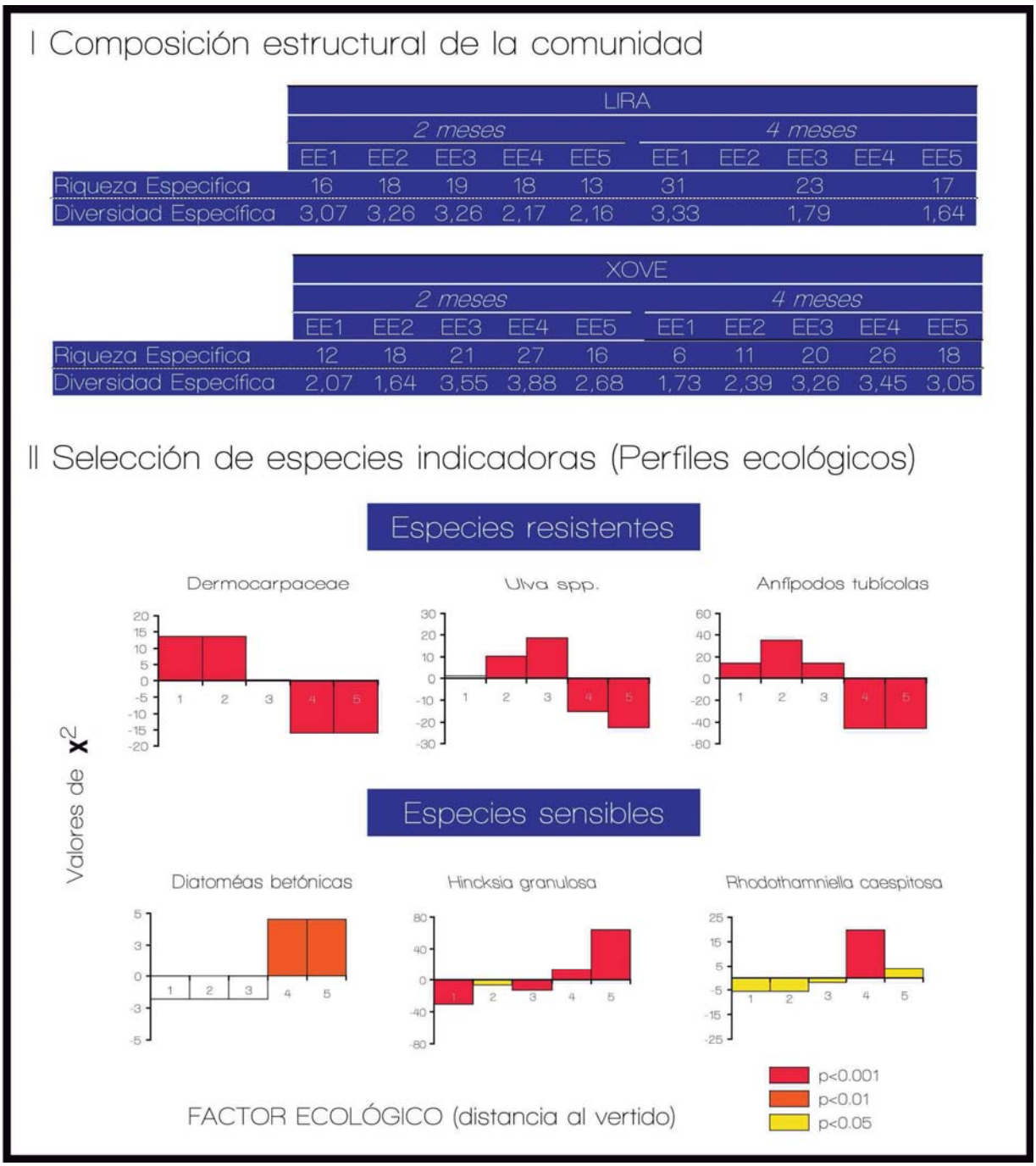


Figura 16. Perfiles ecológicos de especies indicadoras y parámetros macroscópicos de la comunidad colonizadora de sustratos artificiales (fouling) que pueden ser utilizados en la vigilancia de la integridad ecológica de los ecosistemas afectados por los vertidos de las piscifactorías marinas instaladas en tierra (LBMFF).

5.2. Biomarcadores

5.2.1. Biomarcadores moleculares

El análisis de biomarcadores moleculares comprendió el estudio de 3 enzimas de biotransformación (GST, EROD y DBF), 2 enzimas de defensa antioxidante (GPX y GR), y 2 parámetros de daño oxidativo (LPO y daño de ADN) en mejillones nativos, *M. galloprovincialis*, localizados en gradiente respecto al punto de vertido de siete granjas gallegas (Carballeira et al., 2010a) (Figura 19). En cada escenario se establecieron de 3 a 5 puntos de muestreo, en los que se recogieron 10 individuos por punto que fueron diseccionados para obtener las branquias, las gónadas y los hepatopáncreas. Los órganos tuvieron que ser posteriormente agrupados por parejas para tener la cantidad de muestra suficiente para poder realizar el análisis de la batería de biomarcadores.

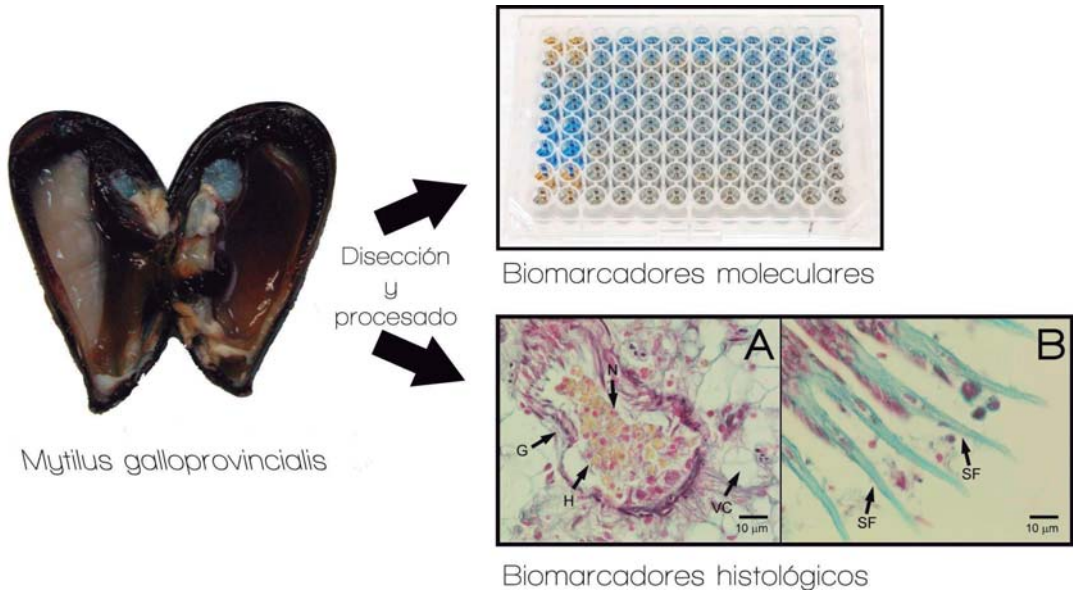


Figura 19. Los biomarcadores cuantificados en mejillones nativos (*M. galloprovincialis*) expuestos a los vertidos de las granjas marinas son un indicador de integridad ecológica. **A.** Ejemplo de fagocitosis hemocítica. **B.** Ejemplo de exfoliación branquial.

Los resultados obtenidos a través del análisis de biomarcadores contenidos en gónada mostraron una enorme variabilidad ya que los niveles de éstos dependen directamente del ciclo o estado de madurez del órgano

reproductor. Por lo contrario, los biomarcadores medidos en las branquias mostraron un patrón claro de variación a lo largo del gradiente espacial.
Las actividades de las enzimas de biotransformación y antioxidantes fueron más bajos en los mejillones cercanos a la salida del vertido en la mayoría de los escenarios, lo que sugiere la adaptación de los organismos al vertido o la inhibición de las actividades enzimáticas causada por la toxicidad de los vertidos. Sin embargo, los niveles de peroxidación lipídica disminuyeron con la distancia, lo que indica la existencia de condiciones de estrés oxidativo. Este tipo de daño oxidativo y de respuestas antioxidantes posiblemente sea inducido por los residuos metabólicos de peces y por sustancias desinfectantes y/o anti-incrustantes.
En general, se observó una mayor sensibilidad de las respuestas enzimáticas medidas en branquias y una relación directa entre el nivel de producción acuícola y las concentraciones de biomarcadores, tanto para branquia como para hepatopáncreas.

5.2.2. Daños histológicos

El análisis de los daños histológicos fue llevado a cabo en *M. galloprovincialis* nativo y en *Venerupis pullastra* Montagu (1803) trasplantada. Esta última especie, conocida como almeja babosa, se utilizó por el interés comercial que presenta su cultivo en la zona de estudio, por la posibilidad de incluirlo dentro de sistemas de cultivo multitróficos y para observar las diferencias en la sensibilidad entre organismos nativos y trasplantados (Carballeira et al., 2011b).
Se observó la exfoliación de los filamentos branquiales de mejillones y almejas y se encontraron algunos casos de fagocitosis hemocítica en gónada y tejido conectivo de almeja (Figura 19A y 19B). Las alteraciones histológicas se contabilizaron semicuantitativamente a través de un índice ponderado de daño (*Weighted Index of Damage*, WID), calculado de la siguiente manera:

$$IPD= [(0 \times \text{Normales (\%)})+ (1 \times \text{Alteración Ligera (\%)})+ (2 \times \text{Alt. Moderada (\%)})+ (3 \times \text{Alt. Grave (\%)})]/100$$

Para cada tipo de daño histológico el IPD de una estación de muestreo fluctúa de 0, cuando ningún individuo presenta ningún daño, a 3 cuando el 100% de los individuos presentan una alteración grave. Las almejas trasplantadas resultaron ser algo más sensibles que los mejillones nativos, pero el coste y los riesgos de emplear organismos trasplantados también es mayor.

Posteriormente este índice fue correlacionado con las señales isótópicas $\delta^{15}N$ contenidas en mejillón y macroalgas nativas. Ambos parámetros se encuentran directamente relacionados, de esta manera cuanto mayor sea la señal $\delta^{15}N$ mayor será el daño histológico (Carballeira et al., 2011b).

La validación de la relación descriptor-efecto, mediante la inclusión de más datos o estudios, permitirá simplificar los aspectos técnicos y mejorar la relación coste-efectividad en el diseño de un plan de monitorización ambiental para este tipo de cultivos.

Artículos del capítulo 5 (ANEXO IV)

Evaluación de la alteración de la integridad ecológica

- *Linking δ15N and histopathological effects in molluscs exposed in situ to effluents from land-based marine fish farms.* **Marine Pollution Bulletin.**
- *Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos de fitoplancton.*
- *Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos in situ de discos de Ulva.*
- *Monitoring chronic effects of land-based marine fish farm effluents at exposed and rocky shores by using biochemical biomarkers in native mussels.* **Enviado.**
- *Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos de colonización de sustratos artificiales (Pendiente de finalización).*

PROPUESTA DE UN PLAN DE VIGILANCIA ADAPTADO A LAS GRANJAS MARINAS INTENSIVAS INSTALADAS EN TIERRA

Actualmente el PVA de los vertidos de las granjas de estudio es llevado a cabo únicamente mediante la medición de parámetros físico-químicos convencionales del agua de entrada, de salida y de diversas zonas próximas al vertido. Sin embargo, para poder apreciar los efectos de la acuicultura es necesario profundizar en las interacciones que el cultivo tiene con el medio. El impacto medioambiental de una piscifactoría marina vendrá fundamentalmente determinado por las características del medio receptor (la profundidad, la intensidad de las corrientes y la naturaleza de los sedimentos son los elementos determinantes para la dispersión, la suspensión o la acumulación del material vertido), del tipo de cultivo (intensivo, semi-intensivo o extensivo; en estanque, jaula, estero,...), la dieta (calidad y métodos de alimentación) y la carga de producción. Por ello, han de plantearse PV específicos a cada zona y hallar los valores de fondo o de referencia previos a la actividad.

Con las conclusiones obtenidas en los distintos estudios de esta tesis, se elaboró una propuesta del plan que será tomada como una primera aproximación, y que es conveniente refrendar y aquilatar con estudios posteriores. De este modo se desarrollará un protocolo de operaciones prácticas preciso y robusto, y se definirán adecuadamente los umbrales interpretativos de los parámetros seleccionados.

El principal objetivo de esta propuesta metodológica es diseñar un plan que sea sencillo en su ejecución, estadísticamente robusto en su dimensionamiento y tratamiento de datos, dinámico en relación a la evolución del medio, estandarizado en cuanto a los métodos analíticos y de obtención de muestras, y uniforme para todas las granjas con características similares (JACUMAR, 2012). Además, el PVA ha de ser económicamente viable, priorizando al máximo la relación coste-información (GESAMP, 2002).

Los nutrientes, DBO (demanda biológica de oxígeno) y los sólidos en suspensión de los efluentes pueden variar en función de la calidad del alimento, estrategia de alimentación, tiempo y ubicación (Tello et al., 2010). Por este motivo, es fundamental centrar la vigilancia ambiental en los períodos de máxima producción (*períodos críticos*). En el caso de las granjas de esta tesis estos períodos suelen ocurrir en verano y principios de otoño (Julio-Octubre) (Figura 20).

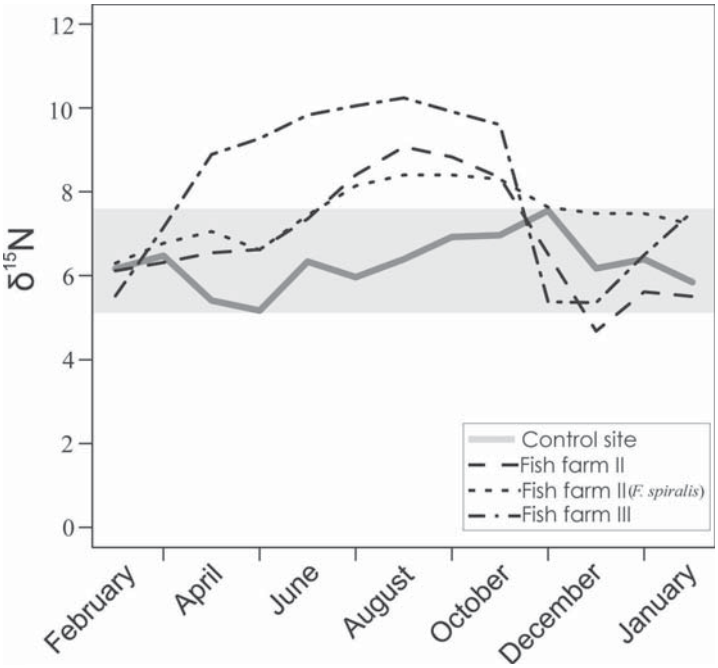


Figura 20. Variación de la señal isotópica $\delta^{15}N$ en macroalgas, del punto control y cercanas a piscifactorías intensivas en tierra, a lo largo del año.

En el PVA integrado y dinámico, propuesto en base a los estudios realizados, también se indican los criterios de calidad ecológica de los parámetros seleccionados como prioritarios.

6.1. *Evaluación de la exposición*

6.1.1. Balance entrada□salida

Prioritariamente se realizarán medidas del $N_{amoniacal}$, pH, y sólidos en suspensión en el agua de entrada y de salida, de muestras compuestas de agua (12h de bombeo diurno). Secundariamente, y por el escaso coste añadido, se podrán determinar también las concentraciones de N_{total} y PO_4^-.

En la Tabla 3 se recogen los parámetros prioritarios y secundarios a analizar en las aguas de entrada (E) y salida (S) de las piscifactorías y se indican los criterios que califican como adecuado, admisible e inadmisible la calidad físico-química de los vertidos.

Tabla 3. Parámetros y criterios de calidad para el balance de agua de entrada y salida de las piscifactorías marinas instaladas en tierra (LBMFF) [Nº: número de datos analizados; E: entrada; S: salida; *Vr*: Valor de referencia para vertidos (Anexo III de la Orden MAM/85/20 08)].

Parámetro	Criterio AUGAS DE GALICIA	Nº	Mediana de los valores observados			ADECUADO	ADMISIBLE	INADMISIBLE
			E	S	S-E			
pH	-	168	8,03 ±0,03	7,73 ±0,06	-0,30 ±0,05	E-S < 0,30	95% casos E - S < 0,35 5% casos E - S < 0,40	>5% casos E - S > 0,40 S < 7,3
N_{NH4} mg/l	- *Vr:1*	54	2,33 ±1,35	2,42 ±1,56	0,12 ±0,21	S-E < 0,12	95% casos S - E < 0,15 5% casos S - E < 0,55	>5% casos S - E > 0,55 S > 4,5
SS mg/l	S-E< 5 *Vr: 25*	180	13 ±20	13 ±27	0,68 ±0,87	S-E < 2,5	95% casos S - E < 2,5 5% caso S - E < 5	>5% caso S - E > 5 S > 75
N_{Total} mg/l	-	157	1,34 ±0,17	1,78 ±0,24	0,29 ±0,13	S-E < 0,45	95% casos S - E < 0,3 5% casos S - E < 0,6	>5% casos S - E > 0,6mg/l S > 4
P fosfatos mg/l	S-E<0,2 *Vr:0,7*	157	0,19 ±0,04	0,24 ±0,05	0,05 ±0,01	S-E < 0,10	95% casos S - E < 0,05 5% casos S - E < 0,15	>5% casos S - E > 0,15 S > 0,35

6.1.2. Medida de la Exposición

Se propone la determinación en la época de máxima producción de la señal isotópica $\delta^{15}N$ en macroalgas nativas recolectadas a modo de gradiente respecto al foco de emisión en la dirección de la corriente dominante. La señal $\delta^{15}N$ es un descriptor de exposición que integra la carga contaminante y la capacidad dispersiva del medio y su uso permite vigilar la intensidad y la extensión del impacto potencial. Se recomienda el empleo de correlaciones con otros parámetros indicadores de impacto para agilizar las labores de vigilancia.

Tabla 4. Objetivos de calidad para el máximo factor de contaminación (FC) de biocidas en macroalgas.

	ADECUADO	ADMISIBLE	INADMISIBLE
FC biocidas en macroalgas	$FC_{MAX} \leq 2$	$2 < FC_{MAX} \leq 5$	$FC_{MAX} > 5$

Adicionalmente se propone el cálculo del Factor de Contaminación (FC) de los organismos del área de influencia de la siguiente manera:

FC= [medidas en organismos *in situ*]/[niveles de fondo]

Este índice mide el grado de bioacumulación de los contaminantes respecto al valor de referencia de la zona de estudio. En la Tabla 4 se recogen los niveles de los FC que categorizan el grado de bioacumulación de los contaminantes.

6.2. *Evaluación ecotóxica de los vertidos*

Se propone una batería mínima de bioensayos con al menos tres especies test pertenecientes a niveles tróficos diferentes (bacteria, microalga e invertebrado) que serán expuestas a muestras compuestas (12h de bombeo) del vertido recogidas durante el período de máxima producción de peces (Julio-Octubre).

El cálculo de la toxicidad de los efluentes (Huella Tóxica, HT) utilizará el EC_{20} obtenido de las curvas dosis-respuesta, y será ponderada por el caudal para obtener el índice PEEP. Por otro lado, los porcentajes de inhibición correspondientes a la dilución 1:4 (v/v) de cada vertido serán utilizados para comprobar si las piscifactorías cumplen los criterios de calidad de los distintos bioensayos (Tabla 5).

Tabla 5. Bioensayos y criterios de calidad ecotoxicológica del agua de salida de las piscifactorías marinas instaladas en tierra (LBMFF) [% Inh= % de Inhibición respecto al control para EC_{20} y una dilución 1:3 del vertido en agua de mar artificial].

	ADECUADO	ADMISIBLE	INADMISIBLE
Test Microtox % Inh	S-E ≤ 20 % Inh	95% casos Inh ≤ 20 % 5% casos Inh ≤ 30 %	> 5% casos Inh > 30%
Test microalgas % Inh	S-E ≤ 20 % Inh	95% casos Inh ≤ 20 % 5% casos Inh ≤ 35 %	> 5% casos Inh > 35 %
Test erizo % Inh	S E ≤ 20 % Inh	95% casos Inh ≤ 20 % 5% casos Inh ≤ 25 %	> 5% casos Inh > 25%
Huella toxica (HT)	HT≤22	95% casos HT ≤ 32 5% casos HT ≤ 43	> 5% casos HT > 43
PEEP	PEEP ≤5,1	95% casos PEEP ≤ 5,5 5% casos PEEP ≤ 5,9	> 5% casos PEEP > 5,9

6.3. *Evaluación de la alteración de la integridad ecológica*

La creación de perfiles ecológicos de las especies obtenidas en el test de colonización permitió seleccionar las especies resistentes (oportunistas) y sensibles al vertido. El estudio de la integridad ecológica es complejo y costoso, por lo que se deberían centrar los esfuerzos en la vigilancia de estas especies.

De manera complementaria, para la vigilancia del estado trófico, se propone la medición (Tett et al., 2007.) de la abundancia (cobertura relativa o biomasa) de las macroalgas oportunistas en la franja intermareal rocosa del área de influencia delimitada por los valores observados de $\delta^{15}N$.

Además, según los resultados obtenidos en el seguimiento ambiental se podrá exigir la realización de estudios histopatológicos en moluscos nativos (p.e. mejillón).

En Tabla 6 se recogen los parámetros y se indican los criterios que califican como adecuado, admisible e inadmisible la alteración de la integridad ecológica dentro del área de influencia de los vertidos.

Tabla 6. Criterios de calidad ecológica del medio receptor afectado por las piscifactorías marinas instaladas en tierra (LBMFF).

	ADECUADO	ADMISIBLE	INADMISIBLE
Abundancia de macroalgas oportunistas			
Δ Cobertura sp oportunistas (intermareal disponible) (%)	Δ ≤ 15	15 > Δ ≤ 30	Δ > 30
Δ Biomasa sp oportunistas (Máximo estacional) (Kg/m^2)	Δ ≤ 1	1 < Δ 1,3	Δ > 1,3
Lesiones histológicas en molusco nativo			
Ind. Ponderado de daño (WID) en *M. galloprovincialis*	WID=0	0< WID ≤ 1	WID >1

6.4. Periodicidad de la vigilancia

La periodicidad de la vigilancia dependerá de la:

- Carga potencial del efluente
- Sensibilidad del medio

Admitiendo que la gestión de las LBMFF (excepto las que recirculan el agua) es similar, la carga potencial del efluente puede ser estimada a partir de la producción anual (t/año), del caudal bombeado (Hm/año) o del consumo energético (Kw/año).

La capacidad de acogida y la sensibilidad del medio ha de ser estimada para cada caso en particular ya que depende de la capacidad dispersiva (hidrodinamismo, batimetría, topografía,...) y de la presencia de poblaciones o hábitats con diferentes niveles de protección.

Suponiendo que se realizó una correcta "elección del sitio" para la instalación de la LBMFF, la periodicidad de la vigilancia se puede diseñar acorde a la carga potencial. En la Tabla 7 se recoge una simulación de la periodicidad de la vigilancia en función de la carga potencial (Tn/año). Como se puede observar en todos los casos se prioriza la vigilancia durante el mes de septiembre (Sp), dentro de la época de mayor producción o cuando el efecto acumulado puede ser más elevado. La idea es que si en el período más crítico los resultados de la vigilancia son satisfactorios, el resto del año lo serían aún más. En el caso de que fuesen insatisfactorios habría que ampliar el período de

Tabla 7. Periodicidad de la vigilancia ambiental de las piscifactorías marinas instaladas en tierra (LBMFF) en función de la carga potencial.

	Carga potencial (Tn.año^{-1})		
	BAJA <500	MEDIA 500-2000	ALTA >2000
Indicadores Balance Entrada - Salida	Trimestral *Mr, Jn, Sp, Dc*	Bimensual *Fn, Mr,My,Jl, Sp,Nv*	Mensual *Fn, Fb Mr,Ab, My,Jn,Jl,Ag,Sp,Oc,Nv,Dc*
Bioensayos ecotoxicidad	Anual *Sp*	Semestral *Sp,Mr*	Trimestral *Mr, Jn, Sp,Dc*
Indicador - descriptor de exposición	Anual *Sp*	Anual *Sp*	Anual *Sp*
Lesiones histológicas en moluscos nativos	Trienal *Sp*	Bienal *Sp*	Anual *Sp*
Integridad ecológica	Trienal *Sp*	Bienal *Sp*	Anual *Sp*

vigilancia.

El PVA ha de ser dinámico por lo que se priorizará más la tendencia que el dato estático. También el PVA debe contemplar la posible reducción de la periodicidad de la medición cuando la actividad de la granja mantiene los parámetros considerados en el plan dentro de los rangos establecidos como los adecuados.

Bibliografía

Aarab, N., et al., 2008. Histopathology alterations and histochemistry measurements in mussel, *Mytilus edulis* collected offshore from an aluminium smelter industry (Norway). Marine Pollution Bulletin. 57, 569-574.

Abrahamson, A., et al., 2007. Gill EROD in monitoring of CYP1A inducers in fish—A study in rainbow trout (*Oncorhynchus mykiss*) caged in Stockholm and Uppsala waters. Aquatic Toxicology. 85, 1-8.

Altinok, I., Grizzle, J. M., 2004. Excretion of ammonia and urea by phylogenetically diverse fish species in low salinities. Aquaculture. 238, 499-507.

APROMAR, 2011. La acuicultura marina en España. APROMAR, Chiclana de la frontera.

Au, D. W. T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Marine Pollution Bulletin. 48, 817-834.

Aubin, J., et al., 2006. Characterisation of the environmental impact of a turbot (*Scophthalmus maximus*) re-circulating production system using Life Cycle Assessment. Aquaculture. 261, 1259-1268.

Blaise, C., Férard, J.-F., 2005. Effluent assessment with the Peep (*Potential Ecotoxic Effects Probe*) index. In: Blaise and Férard (Ed.), Small-scale Freshwater Toxicity Investigations. Springer Netherlands, pp. 69-87.

Borgmann, U., et al., 2001. Identifying cause in sediment assessments: bioavailability and the sediment quality triad. Canadian Journal of Fisheries and Aquatic Sciences. 58, 950-960.

Borja, Á., 2002. Los impactos ambientales de la acuicultura y la sostenibilidad de esta actividad. Boletin Instituto Español de Oceanografía. 18, 41-49.

Burridge, L., et al., 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture. 306, 7-23.

Caeiro, S., 2004. Environmental data management in the sado estuary: weight of evidence to assess sediment quality. University Nova Lisbon Monte da Caparica, pp. 414.

Cairns, J., et al., 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia. 47, 135-171.

Campbell, D. A., et al., 2001. Impact and residence time of oxytetracycline in the sea urchin, *Psammechinus miliaris*, a potential aquaculture species. Aquaculture. 202, 73-87.

Carballeira, A., et al., 2011a. Utilización de perfiles ecológicos para la selección de variables geoquímicas de sedimentos marinos como indicadoras del impacto ambiental generado por los cultivos marinos en mar abierto. XIII Congreso Nacional de Acuicultura, Vol. 13. SEAS, Castelldefels.

Carballeira, C., et al., 2012a. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. Ecotoxicology and Environmental Safety. 78, 148-161.

Carballeira, C., et al., 2011b. Linking $\delta^{15}N$ and histopathological effects in molluscs exposed *in situ* to effluents from land-based marine fish farms. Marine Pollution Bulletin. 62, 2633-2641.

Carballeira, C., et al., 2011c. Influence of salinity on fertilization and larval development toxicity tests with two species of sea urchin. Marine Environmental Research. 72, 196-203.

Carballeira, C., et al., 2010a. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Biomarcadores moleculares en mejillón nativo. XIII Foro dos Recursos Mariños e da Acuicultura das Rías Galegas, Vol. 13. USC, O Grove, pp. 167-176.

Carballeira, C., et al., 2012b. Assessing the toxicity of chemical compounds associated with land-based marine fish farms: The sea urchin embryo

bioassay with *Paracentrotus lividus* and *Arbacia lixula*. Archives of Environmental Contamination and Toxicology. 63, 249-261.
Carballeira, C., et al., 2012c. Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents. Marine Environmental Research. 77, 12-22.
Carballeira, C., et al., 2012d. Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas. Journal of Environmental Monitoring. 14, 1305-1316.
Carballeira, C., et al., 2012e. $\delta^{15}N$ values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms. Journal of Applied Phycology. 1-11.
Carballeira, C., et al., 2010b. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioensayos de fertilidad *in situ*. XIII Foro dos Recursos Mariños e da Acuicultura das Rías Galegas, Vol. 13. USC, O Grove, pp. 201-208.
Casado, C., 2006. Caracterización de material de dragado optimizando un método integrado de evaluación de la calidad ambiental. Chemistry-Physics phD. University of Cadiz, Cádiz, pp. 346.
CETGA, 2005. Desarrollo de un método para minimizar los residuos de los efluentes de plantas acuícolas y su posible valorización. Centro Tecnológico Gallego de Acuicultura, Aguiño, pp. 62.
Helsinki Commission, 2011. Draft helcom recommendation on implementation of whole effluent assessment in the baltic sea region. In: COHIBA Project (Ed.), Actions to limit emissions and discharges of hazardous substances from land-based sources, Vol. 16. Land-based Pollution Group, Dessau.
Conti, E., 1987. Acute toxicity of three detergents and two insecticides in the lugworm, *Arenicola marina* (L.): a histological and a scanning electron microscopic study. Aquatic Toxicology. 10, 325-334.
Cook, E. J., et al., 2006. The influence of caged mariculture on the early development of sublittoral fouling communities: a pan-European study. ICES Journal of Marine Science: Journal du Conseil. 63, 637-649.
Corporation Black and Veatch, 2010. White's handbook of chlorination and alternative disinfectants, John Wiley & Sons, New Jersey.
Costanzo, S. D., et al., 2004. Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. Marine Pollution Bulletin. 48, 514-525.
Costello, M. J., et al., 2001. The control of chemicals used in aquaculture in Europe. Journal of Applied Ichthyology. 17, 173-180.
Crane, M., et al., 2007. Review of aquatic *in situ* approaches for stressor and effect diagnosis. Integrated Environmental Assessment and Management. 3, 234-245.
Chapman, P. M., 2007. Determining when contamination is pollution - Weight of evidence determinations for sediments and effluents. Environment International. 33, 492-501.
Chapman, P. M., et al., 1996. A warning: NOECs are inappropriate for regulatory use. Environmental Toxicology and Chemistry. 15, 77-79.
Chénier, R., 2003. Ecological risk assessment of formaldehyde. Human and ecological risk assessment. 9, 483-509.
Dean, R. J., et al., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: An extensive survey. Environmental Pollution. 145, 84-95.
DelValls, T. A., Chapman, P., 1998. Site-specific sediment quality values for the Gulf of Cadiz (Spain) and San Francisco Bay (USA), using the sediment quality triad and multivariate analysis. Ciencias Marinas. 24, 313-336.
DelValls, T. Á., 2007. Diseño y aplicación de modelos integrados de evaluación de la contaminación y sus efectos sobre los sistemas marinos y litorales y la salud humana. Ministerio de la Presidencia. Secretaría General Técnica. Centro de Publicaciones, Madrid.

Deutsch, B., Voss, M., 2006. Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: Results from *in-situ* incubation experiments. Science of The Total Environment. 366, 799-808.
Dolenec, T., et al., 2007. Nitrogen stable isotope composition as a tracer of fish farming in invertebrates *Aplysina aerophoba, Balanus perforatus* and *Anemonia sulcata* in central Adriatic. Aquaculture. 262, 237-249.
Dosdat, A., et al., 1996. Comparison of nitrogenous losses in five teleost fish species. Aquaculture. 141, 107-127.
Fabregas, J., et al., 1984. Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. Aquaculture. 42, 207-215.
FAO, 2010. The state of world fisheries and aquaculture. Food and Agricultural Organization of the united nations, Rome, pp. 218.
FAO, 2012. El estado mundial de la pesca y la acuicultura. Food and Agricultural Organization of the united nations, Roma, pp. 251.
Fournier, V., et al., 2003. Excess dietary arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss* and turbot *Psetta maxima*. Aquaculture. 217, 559-576.
Galloway, T. S., et al., 2004. Ecosystem management bioindicators: the ECOMAN project – a multi-biomarker approach to ecosystem management. Marine Environmental Research. 58, 233-237.
GESAMP, 1996. Monitoring the ecological effects of coastal aquaculture wastes. FAO, Rome, pp.38.
GESAMP, 1997. Towards safe and effective use of chemicals in coastal aquaculture. Reports and studies, Vol. 65. FAO, Rome, pp. 126.
GESAMP, 2002. Revised GESAMP hazard evaluation procedure for chemical substances carried by ships. Reports and studies, Vol. 64. IMO, London, pp. 39.
Halwart, M., et al., 2007. Cage aquaculture – Regional reviews and global overview. Vol. 498. FAO, Rome, pp. 241.
Hylland, K., 2001. Biological effects of contaminants in pelagic ecosystems- a practical workshop. 2000 Annual Science Conference. ICES, Bruges, pp. 6.
ICES, W., 2002. Report of the working group on biological effects of contaminants. Vol. 2. International Council for the Exploration of the Sea, Gothenburg, pp. 65.
Isidori, M., et al., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of The Total Environment. 346, 87-98.
Isnard, P., et al., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere. 45, 659-669.
JACUMAR, 2012. Propuesta metodológica para la realización de los planes de vigilancia ambiental de los cultivos marinos en jaulas flotantes. JACUMAR, pp. 146.
Jones, A. B., et al., 2001. Assessing ecological impacts of shrimp and sewage effluent: biological indicators with standard water quality analyses. Estuarine, Coastal and Shelf Science. 52, 91-109.
Koleff, P., et al., 2003. Measuring beta diversity for presence–absence data. Journal of Animal Ecology. 72, 367-382.
Kusui, T., Blaise, C., Ecotoxicological assessment of Japanese industrial effluents using a battery of small-scale toxicity tests. Impact assessment of hazardous aquatic contaminants. Arbor Press, Michigan, 1999, pp. 161-181.
Lazard, J., et al., 2011. Evaluation of aquaculture system sustainability: a methodology and comparative approaches, Recent Advances in Fish Farms.
Lin, D. T., Fong, P., 2008. Macroalgal bioindicators (growth, tissue N, $\delta^{15}N$) detect nutrient enrichment from shrimp farm effluent entering Opunohu Bay, Moorea, French Polynesia. Marine Pollution Bulletin. 56, 245-249.
Lojen, S., et al., 2005. $\delta^{15}N$ as a natural tracer of particulate nitrogen effluents released from marine aquaculture. Marine Biology. 148, 87-96.

Lopez, J., et al., 1997. D665/D665a index vs. frequencies as indicators of bryophyteresponse to physicochemical gradients. Ecology. 78, 261-271.
Martin-Diaz, M. L., et al., 2005. Biomarkers and Bioaccumulation: two lines of evidence to assess sediment quality. In: Jay, H. L., Keeley, J., (Eds.), Water Encyclopedia: Water Quality and Resource Development. John Wiley & Sons, New Jersey, pp. 717.
Morales-Caselles, C., et al., 2008. Sublethal responses in caged organisms exposed to sediments affected by oil spills. Chemosphere. 72, 819-825.
Morales-Caselles, C., et al., 2009. A weight of evidence approach for quality assessment of sediments impacted by an oil spill: The role of a set of biomarkers as a line of evidence. Marine Environmental Research. 67, 31-37.
Nasci, C., et al., 1999. Clam transplantation and stress-related biomarkers as useful tools for assessing water quality in coastal environments. Marine Pollution Bulletin. 39, 255-260.
Nier, A. O., 1950. A redetermination of the relative abundances of the isotopes of neon, krypton, rubidium, xenon, and mercury. Physical Review. 79, 450-454.
Pandard, P., et al., 2006. Selecting a battery of bioassays for ecotoxicological characterization of wastes. Science of the Total Environment. 363, 114-125.
Panouillères, M., et al., 2007. Study of the combined effects of a peracetic acid-based disinfectant and surfactants contained in hospital effluents on *Daphnia magna*. Ecotoxicology. 16, 327-340.
Paredes, E., Bellas, J., 2009. Cryopreservation of sea urchin embryos (*Paracentrotus lividus*) applied to marine ecotoxicological studies. Cryobiology. 59, 344-350.
Persoone, G., et al., 2000. New microbiotests for routine toxicity screening and biomonitoring. Kluwer Academic/Plenum Publishers, New York.
Peters, C., et al., 2002. A marine bioassay test set to assess marine water and sediment quality-its need, the approach and first results. Ecotoxicology. 11, 379-383.
Peters, L. D., Livingstone, D. R., 2001. Induction of molluscan cytochrome P450 monooxygenase system as a biomarker of organic pollution in environmental monitoring In: Garrigues, H. B., C.H. Walker, J.-F. Narbonne, (Ed.), Biomarkers in Marine Organisms: A Practical Approach. Elsevier Science, Amsterdam, pp. 572.
Pillay, T. V. R., Kutty, N., 2005. Aquaculture: Principles and Practices. John Wiley & Sons.
Pitta, P., et al., 2005. Mesoscale changes in the water column in response to fish farming zones in three coastal areas in the Eastern Mediterranean Sea. Estuarine, Coastal and Shelf Science. 65, 501-512.
Pitta, P., et al., 2009. Ghost nutrients from fish farms are transferred up the food web by phytoplankton grazers. Marine Ecology Progress Series. 374.
Ramos-Gómez, J., et al., 2011. Validation of *Arenicola marina* in field toxicity bioassays using benthic cages: Biomarkers as tools for assessing sediment quality. Marine Pollution Bulletin. 62, 1538-1549.
Read, P., Fernandes, T., 2003. Management of environmental impacts of marine aquaculture in Europe. Aquaculture. 226, 139-163.
Rey-Asensio, A., et al., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioacumulación de microcontaminantes. In: Rey-Méndez M., L. C., Fernández Casal J., Guerra A., (Ed.), Foro dos recursos mariños e da acuicultura das rías galegas XIII, Vol. 13. USC, O Grove, pp. 201-218.
Riera, P., et al., 2000. Heavy $\delta^{15}N$ in intertidal benthic algae and invertebrates in the Scheldt estuary (The Netherlands): Effect of river nitrogen inputs. Estuarine, Coastal and Shelf Science. 51, 365-372.
Rigos, G., et al., 2004. Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms. Aquatic Toxicology. 69, 281-288.
Robinson, D., 2001. $\delta^{15}N$ as an integrator of the nitrogen cycle. Trends in Ecology & Evolution. 16, 153-162.

Roque D'Orbcastel, E., et al., 2004. Methodological guide for the elaboration of creation of authorization classified installations for the environment protection (CIEP) in marine fish culture for the Corsica area. IFREMER, Brest, pp. 370.

Salomonsen, J., et al., 1999. Modelling advective transport of *Ulva lactuca* (L) in the sheltered bay, Møllekrogen, Roskilde Fjord, Denmark. Hydrobiologia. 397, 241-252.

Sanz-Lázaro, C., et al., 2011. Effects of wild fish and motile epibenthic invertebrates on the benthos below an open water fish farm. Estuarine, Coastal and Shelf Science. 71, 22-30.

Sapkota, A., et al., 2008. Aquaculture practices and potential human health risks: Current knowledge and future priorities. Environment International. 34, 1215-1226.

Sarà, G., 2007. A meta-analysis on the ecological effects of aquaculture on the water column: Dissolved nutrients. Marine Environmental Research. 63, 390-408.

Sarà, G., et al., 2004. Effects of fish farming waste to sedimentary and particulate organic matter in a southern Mediterranean area (Gulf of Castellammare, Sicily): a multiple stable isotope study ($\delta^{13}C$ and $\delta^{15}N$). Aquaculture. 234, 199-213.

Savage, C., 2005. Tracing the influence of sewage nitrogen in a coastal ecosystem using stable nitrogen isotopes. AMBIO: A Journal of the Human Environment. 34, 145-150.

Savage, C., Elmgren, R., 2004. Macroalgal (*Fucus vesiculosus*) $\delta^{15}N$ values trace decrease in sewage influence. Ecological Applications. 14, 517-526.

Selck, H., et al., 2002. Comparing sensitivity of ecotoxicological effect endpoints between laboratory and field. Ecotoxicology and Environmental Safety. 52, 97-112.

Tello, A., et al., 2010. How do land-based salmonid farms affect stream ecology? Environmental Pollution. 158, 1147-1158.

Tett, P., Gowen, R., Mills, D., Fernandes, T., Gilpin, L., Huxham, M., Kennington, K., Read, P., Service, M., Wilkinson, M., Malcolm, S., 2007. Defining and detecting undesirable disturbance in the context of marine eutrophication. Marine Pollution Bulletin. 55, 282-297.

Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: Cytotoxicity and interactions with cytochrome P450 1A. Toxicology In Vitro. 22, 1128-1135.

Tueros, I., et al., 2008. Dissolved metal background levels in marine waters, for the assessment of the physico-chemical status, within the European Water Framework Directive. Science of the Total Environment. 407, 40-52.

Twiddy, D. R., Reilly, P. J. A., 1995. Occurrence of antibiotic-resistant human pathogens in integrated fish farms. FAO Fisheries Report Vol. 514. FAO, Rome, pp. 23-37.

UNEP, 2011. FAO's role for improved integration of fisheries and aquaculture development and management, biodiversity onservation and environmental protection. COFI, Rome, pp. 14.

Valavanidis, A., et al., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety. 64, 178-189.

Van der Oost, R., et al., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology. 13, 57-149.

Viana, I., et al., *In press*. Assessing the toxicity of chemical compounds associated to land-based marine fish farms through the miniaturized bioluminiscence test with *V. fischeri*. Toxicology.

Viana, I. G., et al., 2009. Eliminación de discos fantasmas en bioensayos con *Ulva* sp. XVII Simposio Ibérico de Botánica Criptogámica. Universidade de Lisboa, Tomar, pp. 97-98.

Warwick, R. M., 1993. Environmental impact studies on marine communities: Pragmatical considerations. Australian Journal of Ecology. 18, 63-80.

Wedderburn, J., et al., 2000. The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. Marine Pollution Bulletin. 40, 257-267.

Wilson, M. V., Shmida, A., 1984. Measuring beta diversity with presence-absence data. Journal of Ecology. 72, 1055-1062.

Wipond, K., Dearden, P., 1998. Obstacles to maintaining ecological integrity in pacific Rim national park reserve. Linking protected areas with working landscapes: conserving biodiversity. Science and Management of Protected Areas Association, Wolfsville, pp. 901-910.

Palabras clave

A

Acute toxicity test – Test de toxicidad aguda

Akaike´s information criterion

Algicide – Algicida

Ammonia – Amoniaco

Ammonium – Amonio

Amoxicillin - Amoxicilina

Ampicillin - Ampicilina

ANOVA

Antibiotics - Antibióticos

Antifouling - Anti-incrustante

Antifungal - Antifúngico

Antioxidan enzymes – Enzimas antioxidantes

Antiparasitic - Antiparasítico

Antiseptic - Antiséptico

Anthropogenic - Antropogénico

Area of influence - Área de influencia

Assimilative capacity of the environment – Capacidad de asimilación del medio

B

Background concentration - Concentración de fondo o de referencia

Bathymetry - Batimetría

Benthic ecosystem index – Índice del ecosistema bentónico

Bioaccumulation – Bioacumulación

Bioassay – Bioensayo

Fertility-toxicity bioassay - Bioensayo de fertilidad-toxicidad

Bioavailability – Biodisponibilidad

Biocide – Biocida

Bioconcentration – Bioconcentración

Bioindicator – Bioindicador

Biological oxigen demand - Demanda biológica de oxígeno

Bioluminiscence – Bioluminiscencia

Biomarker – Biomarcador

Biomarker of effect – Biomarcador de efecto

Biomarker of exposure – Biomarcador de exposición

Biomass - Biomasa

Biomonitor

Biomonitoring - Biomonitorización

Biota

Biotest

Biotool - Herramienta biológica

Blastula – Blástula

Branchial exfoliation – Exfoliación branquial

C

Cage-based marine farm – Granja de cultivo marino basada en el uso de jaulas

Carrying capacity of the enviroment– Capacidad de carga del medio

Chemotherapy - Quimioterapia

Chronic toxicity test - Test de toxicidad crónico

Condition index - Índice de condición

Consumer – Consumidor

Trace contaminants - Contaminantes traza

Cytochrome P450 – Citocromo P450

D

D_{665}/D_{665a} Índice de feofetinización

D_{430}/D_{410} Índice pigmentario

Decomposer - Descomponedor

Descriptor of exposure - Descriptor de exposición

Detoxification – Detoxificación

Detritivorous - Detritívoro

Dibenzylfluorescein dealkylase – Dibencilfluoresceina dealquilasa
Disinfectant - Desinfectante
Dispersive capacity of the environment - Capacidad dispersiva del medio ambiente
DNA strand breaks – Roturas de cadena del ADN
Dose-response curve - Curva dosis-respuesta
Drc add-on-package – Paquete drc
Dunnet's test – Test de Dunnet

E

Echinoid - Equinoideo
Ecological/Environmental risk assessment - Evaluación del riesgo ecológico/ambiental
Ecological integrity – Integridad ecológica
Ecosystem - Ecosistema
Ecotoxicity – Ecotoxicidad
Effective concentration - Concentración efectiva
Emerging pollutants - Contaminantes emergentes
Endpoint - Criterio de valoración
Environmental impact - Impacto ambiental
Eutrophication – Eutrofización
Environmental quality objective – Objetivo de calidad ambiental
Environmental monitoring plan – Plan de monitorización ambiental
Environmental Specimen Bank of Galicia - Banco de Especímenes Ambientales de Galicia
Ethoxyresorufin O-deethylase - Etoxiresorufina O-deetilasa
Exogastrula – Exogástrula

F

Filtration rate – Tasa de filtración
Flumequine - Flumequina
Chlorophyll fluorescence - Fluorescencia clorofílica
Formaldehyde - Formaldehído

Fouling community – Comunidad colonizadoras de substratos artificiales
Fouling test – Test de colonización de substratos artificiales
Fungicide - Fungicida

G

Gastrula – Gástrula
Genotoxicity – Genotoxicidad
Glutathione peroxidase – Glutatión peroxidasa
Glutathione reductase – Glutatión reductasa
Glutathione S-transferase – Glutatión S-transferasa

H

Herbicide – Herbicida
Hemocitic phagocytosis - Fagocitosis hemocítica
Histocytopathology – Histocitopatología
Histopathology – Histopatología
Hydrodynamism - Hidrodinamismo

I

Pigmentary index - Índice pigmentario
Input - Entrada, aporte
In situ - En el sitio mismo
Intensive aquaculture - Acuicultura intensiva

K

Kernel density estimation - Estimación de la densidad Kernel

L

Lack-of-fit test – Prueba de la ausencia de ajuste
Land based marine fish farm - Piscifactoría marina instalada en tierra
Line Of Evidence - Línea de Evidencia

Lipid peroxidation – Peroxidación lipídica
Lowest Observed Effect Concentration - Menor concentración con efecto observado
Long-term exposure – Exposición a largo plazo
Long-term response - Respuesta a largo plazo

M

Mann Whitney U-test
MASS package – Paquete MASS
Maximum likelihood value - Valor de máxima verosimilitud
Monitoring - Monitorización
Meso-polyhaline – Meso-polihalino
Micropollutant – Microcontaminante

N

Nitrogen isotopic ratio - Relación o señal isotópica de nitrógeno
No Observed Effect Concentration - Concentración sin efecto observado
Normal distribution - Distribución normal

O

Opportunistic – Oportunista
Osmoregulator – Osmorregulador
Output - Salida, emisario, canal de desagüe
Oxidative stress – Estrés oxidativo
Oxytetracicline - Oxitetraciclina

P

Parametric – Paramétrico
Particle retention efficiency – Eficiencia de retención de partículas
Pathogen – Patógeno
Pearson correlation - Correlación de Pearson
Pesticide - Pesticida

pH

Phase I biotransformation – Biotransformación de fase I

Phase II biotransformation – Biotransformación de fase II

Phytoplankton - Fitoplancton

Piscicide - Piscicida

Pisciculture - Piscicultura

Pluteus – Pluteus

Potential ecotoxic effects probe index - Índice de sondeo de los efectos ecotóxicos potenciales

Trophic potencial - Potencial trófico

Pre-operational state – Estado pre-operacional

Pre-pluteus – Prepluteus

Producer – Productor o productor primario

Net (primary) productivity - Productividad (primaria) neta

R

Reactive oxygen species – Especies reactivas del oxígeno

Retrospective techniques – Técnicas retrospectivas

R-software

S

Screening - Exploración preliminar, cribado

Sediment Quality Triad – Triada para evaluar la calidad de sedimentos

Short-term exposure – Exposición a corto plazo

Short-term response - Respuesta a corto plazo

Statistical significance - Significación estadística

Smatr Package- Paquete Smatr

Sodium hypochlorite – Hipoclorito sódico

Correlación de Spearman - Spearman correlation

Stenohaline – Estenohalino

Streptomycin - Estreptomicina

Sublethal response - Respuesta subletal

Sulfadiazine - Sulfadiacina

Surfactant – Surfactante

Suspended solids - Sólidos en suspensión

T

Toxic effect – Efecto tóxico

Toxicity – Toxicidad

Toxic loading - Carga tóxica

Toxic print - Huella tóxica

Toxic unit - Unidad tóxica

Transplanted organisms - Organismos transplantados

Trophic effect – Efecto trófico

T-student test

Tukey's test

V

Variability - Variabilidad

Verification control - Control de verificación

W

Weighted index of damage - Índice ponderado de daño

X

Xenobiotic – Xenobiótico

Z

Zooplankton – Zooplancton

Glosario de términos

Acclimation – Aclimatación

Adaptación de los organismos a las condiciones en las que se desarrollará el *bioensayo*.

Acute toxicity test – Test de toxicidad aguda

Bioensayo en el que los organismos experimentan una *exposición a corto plazo* (respecto a su fase vital) a uno o varios factores de estrés (sustancias químicas, factores ambientales –temperatura, salinidad, oxígeno disuelto, nutrientes- o muestras ambientales contaminadas - aire, agua, sedimento, agua intersticial). La magnitud de los factores de estrés (e.g. concentración de una sustancia química) suele ser elevada, superior a la que presentan en condiciones naturales, con el fin de provocar respuestas rápidas en los organismos. Dichas respuestas suelen ser evidentes *de visu* y fáciles de cuantificar. Los tests de *toxicidad* aguda tienen como objetivo:

a) Determinar si el factor de estrés puede provocar efectos tóxicos y/o en qué tipo de organismos.
b) Determinar la magnitud del factor de estrés a partir de la cual éste produce efectos tóxicos.

Los parámetros toxicológicos que suelen asociarse a los test agudos se refieren a las dosis o concentraciones letales y subletales (LCx, ECx,) mientras que a los tests crónicos se les asocian el NOEC y el LOEC.

Antifouling AAnti-incrustante

Aditivos de pinturas para barcos y estructuras sumergidas que evitan la fijación de microorganismos, algas y crustáceos.

Antioxidant enzymes – Enzimas antioxidantes

Enzimas encargadas de neutralizar las *especies reactivas del oxígeno*, impidiendo con ello que éstas aumenten su concentración y provoquen *estrés oxidativo*.

Area of influence AÁrea de influencia

Espacio afectado por una actividad en el que pueden ser observables y/o medibles las alteraciones originadas por dicha actividad.

Assimilative capacity of the environment – capacidad de asimilación del medio

Habilidad del medio para integrar una concentración determinada de contaminantes sin que éstos provoquen efectos perjudiciales.

Background concentration AConcentración de fondo o de referencia

Nivel natural de una sustancia química. Concentración de una sustancia en un medio u en un organismo no afectado por la actividad humana.

Benthic – Bentónico

Relativo al bentos o fondo marino.

Benthic ecosistem quality index – Índice de calidad del ecosistema bentónico

Índice que permite evaluar la calidad del sistema bentónico basado en su estructura y/o funcionamiento, así como en las relaciones biológicas.

Bioaccumulation – Bioacumulación

Acumulación de sustancias químicas en organismos que son absorbidas por éstos a través de la respiración, por ingestión o por difusión pasiva a través de la piel al estar en contacto directo con un medio contaminado, ya sea aire, agua, sedimento o agua intersticial. La bioacumulación incluye la entrada corporal de tóxicos por bioconcentración y por biomagnificación.

Bioassay – Bioensayo

Proceso experimental mediante el cual se determinan los efectos adversos de uno o varios estresores (sustancias químicas, factores ambientales o muestras ambientales contaminadas) en organismos indicadores tras un período de exposición variable (exposición a corto o largo plazo) bajo condiciones controladas de laboratorio o bajo condiciones naturales.

ToxicityAfertility algal bioassay ABioensayo de fertilidadAtoxicidad algal

Proceso experimental mediante el cual se evalúa el efecto combinado entre la disponibilidad de nutrientes y de tóxicos en el medio acuático sobre el crecimiento de los productores primarios.

Bioavailability – Biodisponibilidad

Fracción de una sustancia química, en relación a la cantidad total presente en el medio y en la dieta, que puede ser absorbida por un organismo y, por lo tanto, puede penetrar en los tejidos biológicos,

acumulándose en ellos o interactuactuando con los constituyentes celulares.

Biocide – Biocida

Sustancia química o microorganismos destinados a destruir, contrarrestar, neutralizar, impedir la acción o ejercer un control de otro tipo sobre organismos (bacterias, hongos, algas, plantas, animales) considerados nocivos.

Bioconcentration – Bioconcentración

Acumulación de sustancias químicas en organismos que son absorbidas por éstos directamente del medio, es decir, a través de rutas distintas a la ingesta de alimentos o presas.

Bioindicator – Bioindicador

Especie o grupo de especies cuya presencia/ausencia, abundancia o estado permite deducir una cualidad del medio. El estado del bioindicador puede caracterizarse a través de la bioacumulación corporal o de diversos parámetros fisiológicos o bioquímicos (*biomarcadores*).

Bioluminiscence – Bioluminiscencia

Luz emitida por organismos vivos como consecuencia de una reacción bioquímica (enzima-sustrato).

Biomarker – Biomarcador

Parámetro bioquímico, fisiológico, morfológico o conductual cuya variación respecto a una situación de referencia o control permite evaluar la exposición a sustancias tóxicas, su *biodisponibilidad*, así como el efecto adverso de éstas sobre la integridad de los organismos y/o el *ecosistema*.

Biomarker of effect – Biomarcador de efecto

Daños medibles, generalmente irreversibles, provocados por la acción de contaminantes en un organismo.

Biomarker of exposure ABiomarcador de exposición

Cambios medibles que producen en un organismo como respuesta adaptativa o defensiva frente a la presencia de contaminantes. Son indicadores de contaminación.

Biomass ABiomasa

Materia o masa total de seres vivos, expresada en peso por unidad de área o de volumen.

Specific Biomass ABiomasa específica

Materia o masa total de individuos pertenecientes a una determinada especie, expresada en peso por unidad de área o de volumen.

Biomonitor – Biomonitor

Organismo utilizado para realizar el seguimiento de la calidad ambiental.

Biomonitoring ABiomonitorización

Seguimiento de las respuestas biológicas y/o los niveles de contaminantes medidos en organismos con el fin de evaluar posibles cambios en la calidad ambiental de un área determinada, provocados por el inicio de actividades humanas o para asegurar el cumplimiento de valores legislativos de calidad ambiental.

Biota

Conjunto de especies que ocupan un área determinada.

Biotest

Bioensayo.

Biotool AHerramienta biológica

Metodología que usa un organismo sensible a cambios ambientales y que se utiliza para evaluar y monitorizar el *impacto* de actividades humanas sobre los ecosistemas.

Biotransformation ABiotransformación

Conjunto de reacciones químicas a las que está sujeto un tóxico una vez entra en un organismo, con la finalidad de facilitar su eliminación del cuerpo.

Blastula ABlástula

Estado embrionario animal caracterizado por la formación de una masa esférica de células que rodea una cavidad central llena de fluido.

Branchial exfoliation – Exfoliación branquial

Descamación o desprendimiento de las capas superficiales de células de los filamentos branquiales.

Cage-based marine farm – Granja de cultivo marino basada en el uso de jaulas

Actividad acuícola basada en la instalación de estructuras (jaulas, bateas) directamente en el mar (offshore).

Carrying capacity of the environment – Capacidad de carga del medio

Cantidad máxima de contaminantes y organismos que un medio es capaz de soportar de forma sostenible dependiendo de su *capacidad de dispersión* y *asimilación*.

Calciform cells ACélulas calciformes

Glándulas unicelulares que secretan un moco que reviste las células epiteliales de las vías respiratorias y el sistema digestivo.

Chemotherapy – Quimioterapia

Tratamiento basado en la administración de sustancias químicas.

Chronic toxicity tests – Test de toxicidad crónica

Bioensayo en el que los organismos experimentan una *exposición a largo plazo* a uno o varios estresores. La magnitud de los factores de estrés suele ser baja, similar a la que presentan en condiciones naturales, y las respuestas que se estudian suelen ser alteraciones *subletales* (reducción de la eficacia biológica o fitness, anomalías en el desarrollo o histológicas) que aparecen de forma progresiva.

Concentration of subsistence AConcentración de subsistencia

Concentración de cada nutriente para la cual el crecimiento es nulo.

Critical concentration AConcentración crítica

Concentración mínima que satura el crecimiento.

Condition index AÍndice de condición

Relación entre el peso húmedo de los tejidos blandos y el peso de las valvas de moluscos.

Consumer – Consumidor

Animal que adquiere la energía a través del consumo de *productores primarios* (consumidor primario o herbívoro) o de otros consumidores (consumidor secundario o carnívoro).

Trace contaminants AContaminantes traza

Sustancias químicas presentes en el medio en concentraciones muy bajas, de partes por millón o menores. Sinónimo de microcontaminante.

Cytochrome P450 – Citocromo P450

Superfamilia de enzimas encargadas del metabolismo de moléculas endógenas (lípidos, hormonas, esteroides) y de toxinas.

D_{665}/D_{665a} y o D_{430}/D_{410} índices pigmentarios

Índices de estrés fisiológico basados en relaciones de absorbancias pigmentarias (D) a diferentes longitudes de onda (665, 430,410).

Decomposer – Descomponedor

Organismo que adquiere la energía a través del consumo de residuos orgánicos y que transforma en compuestos minerales.

Descriptor of exposure ADescriptor de exposición

Parámetro que proporciona información a cerca del nivel de contaminación presente en un área determinada y que permite determinar la extensión de ésta.

Detoxification – Detoxificación

[1]Eliminación de sustancias tóxicas, es decir, sustancias con capacidad para ocasionar efectos dañinos sobre un organismo.

[2]Proceso de transformación de una sustancia tóxica dirigido a hacer desaparecer su nocividad.

Detritivorous – Detritívoro

Organismo que se alimenta de materia orgánica en descomposición o detrito.

Disinfectant – Desinfectante

Sustancia química que elimina o inhibe la actividad de microorganismos tales como bacterias, virus y protozoos.

Dispersive capacity of the environment A Capacidad dispersiva del medio ambiente

Capacidad para diluir de forma progresiva los contaminantes que son incorporados al medio.

Dose-response curve ACurva dosis-respuesta

Representación gráfica de los efectos causados en un organismo (eje de ordenadas) registrados a distintas dosis de una sustancia química (eje de abcisas).

Ecological/Environmental Risk Assessment A Evaluación del Riesgo Ambiental/Ecológico

Herramienta para la toma de decisiones en la gestión del medio basada en la recopilación de datos químicos, biológicos, ecológicos y ecotoxicológicos que permiten estimar la probabilidad de que las actividades humanas estén desencadenando, o lo hagan en un futuro, efectos adversos significativos sobre los *ecosistemas* y/u organismos constituyentes, y que permiten describir la naturaleza, magnitud y extensión de dichos efectos.

Ecological Niche ANicho ecológico

Rango de condiciones físicas, químicas (incluidos los niveles de contaminantes) y biológicas en el que una especie puede sobrevivir, crecer, reproducirse y mantener una población viable.

Ecosystem – Ecosistema

Unidad compleja y dinámica formada por organismos que conviven en un determinado espacio y el medio en el que habitan, así como por las relaciones de los organismos entre sí y con dicho medio.

Ecotoxicity – Ecotoxicidad

Capacidad de una sustancia química de provocar efectos perniciosos sobre los organismos, poblaciones y comunidades que constituyen un *ecosistema*.

Effective concentration AConcentración efectiva

Concentración de una sustancia química que causa un nivel de efecto determinado, e.g. la concentración efectiva 50 es la concentración que causa el 50% de la respuesta máxima.

Elutriate – Elutriado

Disolución resultante del intercambio de sustancias químicas entre agua y sedimento, tras la mezcla de ambos elementos en una proporción definida y la posterior decantación.

Emerging pollutants AContaminantes emergentes

Sustancias con potencial para dañar la salud de organismos y deteriorar *ecosistemas* que han sido descubiertas en el medio ambiente recientemente o cuyo carácter ecotóxico se desconocía.

Endpoint ACriterio de valoración

Parámetro de respuesta que se mide al finalizar un test para valorar una hipótesis de partida, e.g. en *bioensayos* de *toxicidad*, cuya hipótesis de

partida es que una sustancia química tiene efectos tóxicos sobre la *biota*, el endpoint puede ser la mortalidad de organismos, la inhibición del crecimiento, la degradación de pigmentos clorofílicos, etc.

Environmental impact AImpacto ambiental

Cambios que experimentan los *ecosistemas* en su composición, estructura o funcionamiento, como consecuencia de las actividades humanas, que puede poner en riesgo su integridad y sostenibilidad.

Epiphyte – Epífito

Planta que crece sobre otro organismo y lo utiliza únicamente como soporte.

Eutrophication AEutrofización

Proceso de degradación de un ecosistema por acumulo de biomasa debido al enriquecimiento en nutrientes inorgánicos (nitrógeno y fósforo fundamentalmente) de origen antrópico.

Environmental quality objective – Objetivo de calidad ambiental

Valor máximo aceptable de un parámetro ambiental (concentración de contaminantes y nutrientes, sólidos en suspensión, oxígeno disuelto, etc.) no impuesto por una normativa, y por tanto, no vinculante. Se trata, simplemente, de un valor guía, cuyo cumplimiento es deseable, pero no obligatorio.

Environmental monitoring plan – Plan de monitorización ambiental

Conjunto de medidas, controles e infraestructuras destinadas al estudio de las condiciones ambientales de un *ecosistema* a lo largo del tiempo, cuyo objetivo es predecir la capacidad de dicho *ecosistema* para asimilar *impactos* antropogénicos y anticiparse a posibles cambios en relación a un *estado preoperacional* que redunden en un deterioro de la calidad ambiental.

Environmental Specimen Bank of Galicia ABanco de Especímenes Ambientales de Galicia

Recolección, preparación y almacenamiento de muestras ambientales (bióticas y abióticas) para el seguimiento y el análisis retrospectivo del estado de los ecosistemas.

Exogastrula AExogástrula

Embrión con una morfología anormal que se forma por un aumento cuantitativo del endodermo durante la fase de gastrulación y que no completará su desarrollo.

Filtration rate – Tasa de filtración

En organismos filtradores, como bivalvos, se define como el volumen de agua que pasa a través de las branquias, por unidad de tiempo.

Chlorophyll fluorescence AFluorescencia clorofílica (φPSII)

Relacionada con la capacidad de transporte electrónico del fotosistema II, su medida se puede utilizar como indicador de estrés en organismos fotosintéticos.

Fouling community – Comunidad colonizadoras de substratos artificiales

Conjunto de organismos que crecen sobre la superficie de estructuras artificiales sumergidas, como infraestructuras portuarias, cascos de barcos, tuberías, etc.

Fouling test – Test de colonización de substratos artificiales

Bioensayo basado en el estudio de la comunidad instalada sobre un sustrato artificial sumergido durante un período determinado.

Mesolittoral region AFranja mesolitoral

Zona comprendida entre lo límites de la marea alta y la marea baja, con alternancia entre exposición al aire y al agua. Sinónimo de zona intermareal.

Genotoxicity – Genotoxicidad

Capacidad de una sustancia química para provocar daños en el ADN, uniéndose a él, rompiendo la doble hélice o alterando las enzimas involucradas en la replicación. Las sustancias genotóxicas dan lugar a mutaciones que pueden o no desembocar en cáncer.

Gastrula – Gástrula

Estado del desarrollo embrionario que sigue al de blástula, al sufrir ésta una invaginación. Se compone de tres capas celulares, ectodermo, mesodermo y endodermo.

Hemocytic phagocytosis AFagocitosis hemocítica

Mecanismo de defensa inmunitaria en invertebrados con el que los agentes patógenos (bacterias, virus, parásitos) son eliminados del organismo. Los hemocitos (concretamente los plasmatocitos), que

constituyen la fracción celular de la hemolinfa, rodean con su membrana citoplasmática a los agentes patógenos y los introducen en su interior, donde son posteriormente digeridos.

Hemotoxicity – Hemotoxicidad

Capacidad de una sustancia química para provocar daños en las células sanguíneas.

Histocytopathology – Histocitopatología

Estudio microscópico de los daños en la estructura y funciones de las células y sus repercusiones a nivel tisular.

Histopathology – Histopatología

Estudio microscópico de los daños en la estructura y funciones de los tejidos biológicos.

Hydrodynamism AHidrodinamismo

Movimiento de una masa de agua.

Pigmentary index AÍndice pigmentario

Fórmula matemática que relaciona medidas de pigmentos fotosintéticos, cuyo valor resultante permite evaluar el estado fisiológico de los *productores* primarios.

In situ

En el sitio. Se aplica a las pruebas y análisis que se llevan a cabo en el lugar de estudio (biomonitorización activa), como alternativa a la toma de muestras en campo (biomonitorización pasiva) para su posterior examen en laboratorio.

Intensive aquaculture AAcuicultura intensiva

Sistema de cultivo de organismos (peces, moluscos, etc.) altamente tecnificado, costoso y de elevado rendimiento. Se desarrolla en instalaciones separadas del medio natural. Requiere sistemas de captación, recirculación y/o vertido de agua. Se ejerce un control total sobre el medio y los individuos. Se apoya en la investigación científica para la optimización e invención de técnicas que permitan maximizar la producción al menor coste posible.

Intertidal zone AZona intermareal

Zona comprendida entre lo límites de la marea alta y la marea baja, con alternancia entre exposición al aire e inmersión. Sinónimo de franja mesolitoral.

Land based marine fish farm APiscifactoría marina instalada en tierra

Instalación acuícola intensiva, en la que los peces son cultivados en piscinas artificiales en tierra. La piscifactoría se ubica próxima a la costa, en zonas altamente batidas, de donde bombea agua hacia las piscinas y hacia donde dirigen las aguas residuales.

Line of evidence ALínea de evidencia

Cada una de las ramas o campos que se estudian para obtener pruebas que corroboren una hipótesis de partida.

Lipid peroxidation – Peroxidación lipídica

Degradación de los lípidos de membranas celulares a causa del ataque oxidativo de especies reactivas de oxígeno y radicales libres.

LongAterm exposure – Exposición a largo plazo

Exposición de organismos a un factor de estrés durante un período de tiempo significativo en relación a la esperanza de vida del organismo, el cual puede variar de minutos a años.

LongAterm response – Respuesta a largo plazo

Efecto que experimentan los organismos como consecuencia de una exposición a largo plazo (respecto a la duración de su fase vital) a un factor de estrés y cuya aparición es progresiva.

Metabolite – Metabolito

Molécula que se genera como producto de la transformación de otra molécula durante el metabolismo.

MesoApolyhaline – MesoApolihalino

Variable entre mesohalino (salinidad entre 5 ‰ y 18 ‰) y polihalino (salinidad entre 18 ‰ y 30 ‰.

Monitoring – Monitorización

Seguimiento de las condiciones ambientales de un *ecosistema* con el objetivo de detectar cambios potencialmente perjudiciales provocados por actividades humanas. La detección temprana de alteraciones en un *ecosistema* permite poner en marcha medidas correctoras que impidan su deterioro.

Micropollutant AMicrocontaminante

Contaminante presente en el medio en muy bajas concentraciones. Sinónimo de contaminante traza.

Neurotoxicity – Neurotoxicidad

Capacidad de una sustancia química para alterar el funcionamiento normal del sistema nervioso y de dañar los tejidos nerviosos.

Nitrogen isotopic ratio ARelación o señal isotópica de nitrógeno $\delta^{15}N$

Es el valor obtenido del cociente entre las concentraciones de los isótopos de nitrógeno $^{15}N/^{14}N$ y su comparación con la del aire (medido en ‰).

Normal distribution ADistribución normal

Distribución de probabilidad que da como resultado una curva acampanada y simétrica conocida como campana de Gauss.

Opportunist – Oportunista

Especies que pueden existir en entornos muy variables, ya que se adaptan fácilmente y tienen una gran capacidad para colonizar con rapidez. Frente a un cambio significativo en un *ecosistema* pueden llegar a predominar frente a especies más especializadas, debido a que estas últimas no son capaces de responder eficazmente a los cambios.

Osmoregulator – Osmorregulador

Organismo capaz de mantener estable la concentración de iones en el medio interno, independientemente de la existente en el medio externo, a través del control de la cantidad de líquidos corporales.

Oxidative stress – Estrés oxidativo

Primacía de la producción de *especies reactivas del oxígeno* y sus efectos frente a la capacidad de un organismo para eliminarlas y contrarrestar dichos efectos.

Parametric – Paramétrico

La estadística paramétrica es aquella que trata datos cuya distribución es normal.

Parental or parent compound

Compuesto original. Compuesto cuya transformación dará lugar a uno o varios *metabolitos*.

Particle retention efficiency – Eficiencia de retención de partículas

En organismos filtradores, como bivalvos, es la eficiencia con la que partículas de un determinado tamaño son atrapadas por el mucus que cubre la pared interior de la faringe. Se calcula midiendo la disminución de la concentración de partículas en un determinado volumen, por unidad de tiempo.

Persistent – Persistente

De difícil degradación y, en consecuencia, con tiempos de permanencia prolongados.

pH

Potencial de hidrógeno. Indica la concentración de iones de hidrógeno [H^+] en una solución, y con ella, la acidez o alcalinidad de dicha solución.

Phase I biotransformation – Biotransformación Fase I

Fase inicial del proceso de transformación de un *xenobiótico* llevada a cabo por el *citocromo P450*, consistente en reacciones de oxidación, reducción e hidrólisis a través de las cuales la solubilidad del compuesto aumenta facilitando su posterior eliminación. En ocasiones, este proceso puede dar lugar a *metabolitos* más tóxicos que el *xenobiótico* original (*parent compound*).

Phase II biotransformation ABiotransformación Fase II

Comprende las reacciones de conjugación en las que moléculas polares endógenas se unen a moléculas xenobióticas, que pueden o no haber sufrido previamente la biotransformación de la fase I. La inclusión de un grupo polar aumenta la solubilidad del *xenobiótico* y facilita su excreción.

Pluteus

Estadío larvario de los equinodermos, caracterizado por una morfología pseudocónica, en cuyo vértice se fusiona un haz de espículas, y en la parte inferior se desarrollan una serie de brazos. La larva o *pluteus* es nadadora, planctónica y se alimenta de fitoplancton.

Potential Ecotoxic Effects Probe index A Índice de Sondeo de los Efectos Ecotóxicos Potenciales

Valor numérico resultante de una fórmula matemática en la que se integra el potencial tóxico de vertidos de aguas residuales (determinado mediante *bioensayos* que representan distintos niveles biológicos y distintos tipos de efectos) y su flujo o caudal. Permite evaluar y comparar

la peligrosidad de vertidos de diferente naturaleza bajo el criterio de aditividad.

Trophic potencial APotencial trófico

Capacidad de un vertido de aguas residuales de acrecentar el estado trófico de un sistema al aumentar la disponibilidad de nutrientes.

PreAoperational state – Estado preAoperacional

Características propias de un *ecosistema* antes de verse afectado por una nueva actividad humana.

Pre*Apluteus*

Hace referencia a todas las fases de desarrollo embrionario anteriores a la formación de la larva o *pluteus*.

ProAbiotic – Probiótico

Microorganismos que no pierden su actividad al ser ingeridos y que contribuyen al equilibrio de la flora intestinal y a fortalecer el sistema inmunitario.

Ecological process AProceso ecológico

Serie de cambios físicos, químicos o biológicos que tiene lugar en un *ecosistema* como consecuencia de la interacción entre los organismos o entre éstos y el medio. Esto incluye procesos ecotoxicológicos como la eutrofización, la bioacumulación de contaminantes, etc.

Producer AProductor o productor primario

Organismo que sintetiza materia orgánica a partir del dióxido de carbono, a través de fotosíntesis (usando energía lumínica) o quimiosíntesis (usando energía de reacciones de oxidación-reducción).

Net primary productivity AProductividad primaria neta

Es el resultado del balance entre la energía que se fija a través de la fotosíntesis, menos aquella que es consumida durante la respiración.

Reactice oxygen species – Especies reactivas del oxígeno

Moléculas, iones y radicales libres que contienen oxígeno ($^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$, OH^-), altamente reactivas que aparecen como subproducto del metabolismo oxidativo natural, pero también por la acción y el metabolismo de tóxicos o por la exposición a radiaciones ionizantes. Estas especies pueden aumentar su concentración (*estrés oxidativo*) y

reaccionar con facilidad con componentes celulares llegando a provocar daño celular.

Retrospective techniques – Técnicas retrospectivas

Aquellas técnicas empleadas para definir el *estado pre-operacional de un ecosistema.*

Screening ACribado

Exploración preliminar en un proceso de investigación en la que se seleccionan los lugares/contaminantes/concentraciones de mayor interés y que permite realizar un proceso de cribado de la información inicial y de priorización de los recursos.

Sediment Quality Triad – Triada para evaluar la calidad de sedimentos

Evaluación de la calidad de sedimentos basada en el enfoque del peso de la evidencia (weight of evidence), que incluye tres líneas de evidencia: la concentración de contaminantes en el sedimento, la *toxicidad* del sedimento y la estructura de la comunidad bentónica.

ShortAterm exposure – Exposición a corto plazo

Exposición de organismos a un factor de estrés durante un período corto de tiempo en relación a la duración de su fase vital (alrededor de un 10%).

ShortAterm response – Respuesta a corto plazo

Efecto que experimentan los organismos tras una *exposición a corto plazo* a un factor de estrés.

Statistical significance ASignificación estadística (valor p)

En una prueba estadística, es la probabilidad de que una hipótesis de partida (e.g. la media de dos conjuntos de muestras son iguales) sea cierta. El valor umbral para aceptar o rechazar la hipótesis se establece convencionalmente en 0.05 o 0.01. Cuando al comprobar la veracidad de la hipótesis resultan valores de p superiores al umbral, ello indica que la hipótesis se acepta (e.g. las medias son iguales). Si se obtienen valores de p inferiores al umbral marcado, ello indica que la hipótesis se rechaza (e.g. las medias son distintas).

Sinergystic – Sinérgico

El efecto sinérgico de dos o más sustancias es aquel que resulta de la interacción entre ellas, y se caracteriza por ser mayor que el efecto que produciría cada una de ellas por separado o por la suma de éstos.

Stenohaline – Estenohalino

Organismo que tolera estrechos rangos de salinidades. Opuesto a eurihalino.

Sublethal response ARespuesta subletal

Alteraciones a nivel bioquímico, tisular o fisiológico que se producen en un organismo expuesto a uno o varios factores de estrés.

Supernatant – Sobrenadante

Tras un proceso de centrifugación, es la fracción líquida que queda por encima de otra que precipita.

Surfactant – Surfactante

Sustancia química con propiedades específicas para la limpieza, humectantes, dispersantes, emulsionantes, espumantes y antiespumantes, que forman parte de la composición de una amplia gama de productos químicos como detergentes, pinturas, adhesivos, ceras, pesticidas, cosméticos, etc…

Teratogenic – Teratogénico

Agente capaz de alterar el desarrollo embrionario causando malformaciones congénitas anatómicas, estructurales, funcionales o metabólicas.

Toxicity – Toxicidad

Capacidad de una sustancia química para dañar a un organismo a cualquier nivel de organización biológica, desde el bioquímico hasta el fisiológico, en cualquier etapa de su desarrollo.

Toxic loading ACarga tóxica

Huella tóxica de un vertido en relación con su caudal.

Toxic print AHuella tóxica

La *toxicidad* de un vertido en función de los resultados obtenidos en varios *bioensayos*.

Toxin AToxina

Sustancia tóxica sintetizada por algunos seres vivos, principalmente microorganismos.

Trophic effect – Efecto trófico

Alteración de las condiciones fisicoquímicas y de la estructura de las comunidades biológicas de un *ecosistema* causada por el aumento de la concentración de nutrientes.

Transplanted organisms AOrganismos transplantados

Organismos (nativos o exóticos) que son trasladados a un hábitat que no es originariamente suyo, pero que es apto para su supervivencia, con el fin de estudiar los efectos que les provoca un factor de estrés presente en el nuevo medio (biomonitorización activa).

Variability AVariabilidad

Es la dispersión de datos, es decir, indica si los valores de una variable están muy alejados del valor medio y, por lo tanto, si son parecidos o si varían mucho entre ellos.

Verification control AControl de verificación

Prueba cuyo resultado se conoce y está certificado. Se utiliza para confirmar la validez de las medidas y/o la correcta calibración de los equipos de análisis.

Weighted index of damage AÍndice ponderado de daño

Índice con el que se cuantifica el nivel de alteración histológica de una muestra de acuerdo con la frecuencia con la que determinadas lesiones ocurren.

Xenobiotic AXenobiótico

Sustancia ajena (extraña) al cuerpo que, sin ser producida por éste, puede estar presente en un organismo. Se consideran xenobióticos a fármacos, drogas, y contaminantes ambientales como pesticidas, hidrocarburos aromáticos policíclicos, bifenilos policlorados, furanos, dioxinas, y otros compuestos de origen sintético. También pueden considerarse xenobióticos compuestos de origen natural y metales, siempre que hayan sido movilizados por la acción del hombre. En un sentido más genérico se entiende por xenobiótico la sustancia de origen sintético por oposición a homobiótico, la que es de origen natural.

Anexo I

Journal of Environmental Monitoring

Dynamic Article Links

Cite this: *J. Environ. Monit.*, 2012, **14**, 1305

www.rsc.org/jem

CRITICAL REVIEW

Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas

C. Carballeira,*[a] J. Ramos-Gómez,[b] M. L. Martín-Díaz,[ac] T. A. DelValls[a] and A. Carballeira[b]

Received 17th October 2011, Accepted 20th February 2012
DOI: 10.1039/c2em10839a

The increase in aquaculture activities in the last few decades has not been accompanied by a corresponding increase in environmental controls and regulations. In this context, the application of environmental monitoring plans (EMPs) has become necessary to assess the environmental impact associated with fish farming wastes. The objective of this review paper is to evaluate the suitability of experimental and analytical procedures as monitoring tools for inclusion in EMPs for intensive land-based marine fish farms (LBMFFs). The strong hydrodynamics and, in particular, the lack of sediment on the rocky coasts where LBMFFs are usually located, greatly limit the monitoring tools that can be used. We propose EMPs that employ a weight-of-evidence approach to evaluate: contamination, trophic and toxic effects, and ecological integrity. Laboratory tests, *in situ* bioassays and field surveys of local species are presented as key tools for assessing the impact of LBMFFs on ecosystems. The $\delta^{15}N$ signal along a spatial gradient is proposed for evaluating exposure to contaminants. Trophic effects can be determined by growth of transplanted macro- and microalgae. Toxic effects can be evaluated by responses at different levels of biological organization, including biochemical and histological changes, physiological alterations and survival, in species from different trophic levels. Fouling tests and analysis of community structures are recommended for assessing ecological integrity. This review contributes to the development of environmental controls for intensive LBMFFs, and for other activities that discharge wastewater to rocky shores.

1. Introduction

Fish farming activities should be compatible with other coastal activities that exploit natural resources. Thus, good environmental practices must be applied in order to guarantee sustainability.[1] One of the major problems associated with aquaculture is the detrimental effects on the environment, resulting from the release of excessive amounts of nutrients (which may affect the trophic balance) and synthetic chemical products (which may have toxic effects) to natural bodies of water.[2] This problem is aggravated by the fact that fish farming is expanding at a much faster rate than the knowledge of its effects on ecosystems, and the pace at which regulations and legal requirements are being enforced. This is clearly illustrated by the lack of ecotoxicological research and legislation relating to aquaculture, particularly land-based marine aquaculture. Read and Fernandes[2] carried out a review addressing how the impacts associated with aquaculture are managed, and pointed out the need for specific actions in the aquaculture sector in order to ensure full

[a]*UNITWIN/UNESCO/WiCoP, Physical Chemistry Department, University of Cádiz, 11510 Puerto Real, Cádiz, Spain. E-mail: carlos.carballeira@uca.es*
[b]*Ecotoxicology, Ecology Department, Faculty de Biology, University of Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, Spain*
[c]*Andalusian Center of Marine Science and Technology (CACYTMAR), Campus Universitario de Puerto Real, 11510 Puerto Real, Cádiz, Spain*

Environmental impact

Land-based marine aquaculture is generally considered a non-polluting activity. This is because there is a gap of knowledge about the effects that land-based marine fish farm (LBMFF) effluents may cause to the aquatic systems. Hydrodynamics and lack of fine-grained sediments characterizing nearby coastlines where LBMFFs are located make the use of conventional monitoring methods problematic. The information provided by chemical analyses (current monitoring measures) is not sufficient to explain the potential effects of aquaculture on ecological processes in aquatic systems. This review paper discusses the development of an environmental monitoring plan adapted for land-based marine fish farms, carefully considering the diverse interactions between this type of aquaculture and the surrounding environments.

integration of environmental protection requirements, as well as for further research on the relationship between aquaculture and the environment.

The lack of knowledge about the potential impacts associated with land-based marine fish farms (LBMFFs) makes high quality environmental surveillance essential and the development of Environmental Monitoring Plans (EMPs) imperative. EMPs should integrate the measurement of chemical and ecotoxicological parameters, which provide information about the level of contamination, the bioavailability of contaminants and the effects of contamination on biota. EMPs should also be developed according to a conceptual model that facilitates the integration of results in order to obtain a holistic perspective of the environmental status of areas affected by LBMFF effluents.

The objectives of this review are to describe the factors involved in the design of an EMP, to establish basic guidelines for the design of EMPs, and to propose a specific EMP for LBMFFs located at exposed and hard bottom coastal areas. In order to fulfil these aims, abundant scientific literature was reviewed, contaminants associated with LBMFFs were investigated, and tools for environmental surveillance, including experimental methods of obtaining data and parameters describing alterations in ecosystems were evaluated according to their simplicity, reproducibility, reliability and cost-effectiveness.

2. Contamination from LBMFFs

The main impact of LBMFFs on the aquatic systems is caused by the release of wastewater, containing metabolic waste produced by fish and by the application of synthetic chemical products to fish growth and facility maintenance (Table 1). Input of contaminants *via* the discharge of such waste may trigger a series of biological changes that can have negative effects on ecosystems. The escape of farmed fish may also cause introduction of invasive or unwanted species into the natural environment (biological contamination), with possible ecological costs.

2.1. Metabolic and feed waste

It has been estimated that around 23% of the food supplied to fish is not consumed, and is directly released to the environment, 63.5% is eaten by the fish and partially excreted, and 13.5% is released as feces.[3] This particulate waste, together with metabolic waste excreted by fish, affects the trophic balance in the aquatic environment as it is a primary source of nutrients. Mineralization of organic matter produces phosphates and nitrogen species (NH_4^+, NH_3, NO_2^-, NO_3^-), some of which (NH_4^+ and NO_3^-) are assimilated by aquatic primary producers.[4] Discharged organic matter can thus favour primary biological production and contribute to nutrient enrichment of nearby marine waters. Nevertheless, large increases in the concentrations of nitrogenous compounds may be detrimental, as nitrogen is a limiting nutrient in coastal and estuarine environments.[5] If the concentrations of nitrogenous compounds are maintained at high levels as a result of chronic and extensive discharge of organic matter, it may boost primary producers, finally leading to eutrophication. Eutrophication produces hypoxia and the appearance of toxic compounds, such as hydrogen sulfide.[6] In addition, some of the primary producers favoured may be toxic algae. All of this may cause massive kills of aquatic organisms and a general impoverishment of the biota and a decrease in biodiversity.[5]

Nutrients may also have toxic effects. Inorganic forms of nitrogen have been found to harm aquatic organisms, including bacteria, algae, invertebrates and fish.[5,7]

Organic particulate waste is known to have negative effects on aquatic systems by modifying the chemical and biochemical properties of the water column and the sediment, particularly those involved in food availability, which determine bacterial and primary production.[8,9] Particulate waste can also alter the composition of organisms suspended in the water column, as well as fouling organisms and benthic communities,[9,10] *e.g.* by attracting opportunistic carnivorous and detritivorous species. In both the water column and sediments, organic matter acts as a trap for synthetic lipophilic compounds (including several pharmaceuticals, organochlorine pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls), thus increasing the risk of incorporation of toxic substances into the trophic chain,[11] *e.g.* through filter feeders and detritivorous organisms.

A system for eliminating toxic forms of nitrogen in LBMFFs, by converting ammonia and nitrate into nitrogen gas *via* aerobic and anaerobic nitrification and oxidation, has been created.[12] An easier way of eliminating particulate waste is to use filters. An economic and efficient rotary filter of mesh size 60 μm would collect 40% of suspended solids (SS). However, the main problem with this approach is finding a use for the sludge produced.[3] Other sustainable technologies are emerging in order to mitigate the impact of the input of organic matter into aquatic systems. Such methods involve the elimination of organic matter by using algae and filter-feeding species.[13–15]

Table 1 Summary of substances released from fish farms to the environment

Waste	Frequency	Type of waste
Particulate waste		
Faeces	Continuous	Animal origin
Unconsumed food	Continuous	
Dissolved waste		
(*Excretion*)	Continuous	Animal origin
Treatment products		
Antifouling (TBT, Zn, Cu, …)	Continuous	
Disinfectants and cleaning products	Occasional	Anthropogenic
Zoosanitary products (antibiotics, anaesthetics, …)	Occasional	
Transmissible pathogens to wild life		
Faecal coliforms, *E. coli*, faecal streptococci, …	Occasional	Anthropogenic

2.2. Anthropogenic products

Few studies have considered synthetic chemicals when assessing the impact of aquaculture activities.[16] However, human-made products, such as biocides and sanitary products, are commonly used in aquaculture facilities and are known to have toxic effects on living organisms.[2] Nevertheless, data on the specific type of products used and released to the aquatic systems are scarce, owing to the insufficient regulations and controls within the sector, and a lack of transparency in fish farm management.

Drugs are used prophylactically and therapeutically to maintain optimal fish health[17,18] and to avoid animal stress during transport and manipulation. Hormones may be supplied as trace constituents of fish food,[19] applied by immersion, intramuscular injection or genetically transferred into fish. Hormones are directly involved in growth and reproduction cycles.[20] Pesticides (bactericides, herbicides, fungicides), antifouling products, disinfectants and detergents are used to prevent disease outbreaks and proliferation of algae, and for cleaning and disinfecting ponds.[21] Trace concentrations of metals, pesticides, polybrominated diphenyl ethers, polychlorinated biphenyls and polybrominated biphenyls have also been detected in fish food.[22,23] Drugs and biocides used in pond maintenance are probably the products associated with the greatest environmental risk because of the frequency of use and the large quantities used.

2.2.1. Antibiotics and antiparasitics. Antibiotics and antiparasitics are used respectively to fight fish diseases caused by bacteria, and by nematodes, cestodes, trematodes, infectious protozoa and amoebae.[24] The use of these products involves an environmental risk, since surplus product is released into the environment[25] directly or *via* unmetabolised and/or uneaten medicated feed, leached drugs, renal excretion and branchial secretions.

The persistence of many antibiotics and their metabolites in the marine environment is not known[26] and their impact on ecosystems is still difficult to determine.[27] There is a potential risk that local microbial communities will be modified, and in fact, high rates of resistance to several antibiotics have been observed in sediment bacteria below fish farms.[18,28]

Antiparasitics may affect wild species that are taxonomically unrelated to the target species, particularly crustaceans.[29] The use of vaccines, immunostimulants and genetically altered (disease-resistant) species to fight diseases and infections, is emerging in place of the use of antibiotics. Nevertheless, the use of these alternatives is still limited because of technical and economic issues and/or low effectiveness. Nowadays, the most feasible method of minimizing antibiotic input and impact in natural water bodies consists of avoiding prophylactic treatment, applying effective and narrow spectrum antibiotics, adopting withdrawal periods and avoiding discharge of toxic chemicals into natural bodies of water.[17]

2.2.2. Pesticides, disinfectants, antifouling products and detergents. Detergents and chemical products with biocidal actions (pesticides, disinfectant and antifouling agents) are used daily at high concentrations on LBMFFs, for hygiene, to prevent outbreaks of infectious diseases and for general maintenance of facilities.

The most commonly used biocides include chlorine products, aldehydes and their derivatives. Sodium hypochlorite is a strong, inexpensive oxidant used to disinfect water, tanks and equipment. This compound is toxic to aquatic life at very low concentrations and can oxidize organic matter, producing a mixture of organochlorine compounds, including mutagenic and carcinogenic substances, such as trihalomethanes and chlorinated hydrocarbons.[30]

Formaldehyde is used as an antiparasitic agent, as a biocide and to neutralize ammonia from fish ponds.[31] At high concentrations or after long periods of exposure, formaldehyde solutions are toxic to certain aquatic species, ranging from plankton to fish.[32] Formaldehyde may cause neurotoxic, haematotoxic, genotoxic and carcinogenic effects on ecosystems.[33] The toxicity of detergents differs depending on whether they are anionic, non-ionic or cationic. However, even at low concentrations they may alter the structure of membrane proteins and cause progressive permeabilization and lysis of cells.[34] The toxic effects of certain disinfectants and detergents may also be synergistic.[35] There are no effective alternatives to disinfectants, as although ultraviolet radiation is efficient against bacteria there are technical problems associated with its use (the efficacy is affected by turbidity and dissolved substances), it is not effective against chemicals and viruses, and it does not provide residual protection, so that a secondary disinfectant may be required.[30]

2.3. Biological contamination

Biological contamination can be defined as the unintended release of non-native species or genetically modified species and pathogens from LBMFFs into natural ecosystems.

Biological contamination may lead to genetic contamination when escaped fish reproduce with wild fish, resulting in either more robust fish, which could disrupt the environment, or poorly adapted fish, which could lead to gradual extinction of the species.[36] Negative effects of introduced fish on the genetics of natural populations will increase with the number of generations in captivity.[37] Genetically modified escaped fish can inhabit areas that are unfavourable for wild fish, and thus displace other species. They may alter the trophic balance by increasing predation rates, and may introduce and spread particular diseases and parasites.[38]

The features of the LBMFF culture facilities make the risk of biological contamination of wild populations very low. Nevertheless, regulatory actions should be complemented with management based on preventive measures.[39,40]

3. General guidelines for the design of an EMP

An EMP can be defined as a set of measures, controls and infrastructures aimed at studying environmental conditions in an area over time, in order to predict the capacity of the environment to assimilate anthropogenic impacts, and to anticipate possible changes in environmental quality, taking into account the pre-operational state of the environment.

There are three essential stages to consider in EMPs: (a) prior or pre-operational monitoring, which considers the natural state of the environment before the disturbance occurred, by prior measurement or estimation of background regional levels, (b) effect monitoring, which begins once early alterations have appeared, and aims to prevent detrimental impacts from becoming massive and irreversible, and (c) verification of control, which guides environmental management in relation to the objectives established by current law, and observes the degree of compliance with quality standards.

The specific details of an EMP designed for a fish farm must be in accordance with the farm production volume and the foreseeable impact on the environment, *i.e.* it should be adjusted to the exploitation volume–environmental impact ratio. After an extensive review, Sarà[10] concluded that available research on the

impact of aquaculture in the water column is somewhat defective and insufficient to support or exclude any potential effects on ecological processes in the aquatic environment. For this reason, only those parameters that provide information at an affordable cost should be considered in monitoring.[41] The information obtained should contribute to detecting effects that alter the ecosystem and should not merely describe local situations.[42] For example, to assess the risk of eutrophication, it is better to use a response parameter such as a fertility bioassay, than to measure a large number of physicochemical properties of water.[10] In this sense, the establishment of background levels and characterization of the pre-operational state become important for discriminating changes involving potential ecological damage and natural variability in local conditions.[43]

The impact of aquaculture installations, regardless of the type of exploitation (cage-based or land-based), dissipates with distance from the facilities. An area of influence should be delimited and the parameters selected to evaluate the environmental effects should be sampled and measured according to a non-linear gradient from the discharge outlet (land-based farms) following the direction of the prevailing current.[8,44]

For accurate assessment of the potential ecological effects of the discharge of LBMFF effluent, both spatial and temporal variations in the contaminant levels and monitoring variables must be considered. The concentration of nutrients and toxicants in the farm surroundings, and therefore, the potential of the aquaculture activity to damage the environment, mainly depend on the relationship between the assimilation and dispersive capacity of the environment.[45–47] Although fish farm effluents are known to exert local effects, the area of influence (*i.e.* the distance at which the physical, chemical and biological characteristics of the environment are no longer significantly different from those in a control area) of the effluents changes over time.[16] Such variations depend on factors that affect the assimilative capacity of the environment, such as the sea and weather conditions (prevailing currents, hydrodynamics, storm frequency, wind velocity), and also on the farm production cycle or the frequency of the use of chemical products. The assimilative capacity can be defined as the ability of a body of water to purify itself of contaminants without having negative effects on ecosystem health.[46] In order to assess the maximum effects associated with LBMFFs and to minimize costs, surveillance of aquaculture activities should focus on critical periods, when the area of influence is expected to be more extensive and/or the dispersive capacities of the environment are diminished. The application of waste dispersal models and their potential effects (*e.g.* DEPOMOD,[48] CSTT[49]) may help predict such critical periods.

EMPs should also be operationally flexible. During observation of the affected ecosystems, the addition or replacement of measurement parameters, relocation of monitoring stations, and modification of sampling intensity may be necessary, depending on the spatio-temporal variations in contamination and changes in aquaculture technology, which may lead to changes in the effluent composition (*e.g.* replacement of bioactive compounds).

4. Considerations for EMPs for LBMFFs

Protocols for monitoring the effects of LBMFFs on natural water bodies are practically non-existent. The available regulations, methodological guidelines and EMPs for aquaculture activities in Europe mainly refer to cage-based aquaculture.[1,50–54] These monitoring and management tools cannot be applied to LBMFFs in most cases because of the different idiosyncrasies of cage-based and land-based aquaculture. Cage-based aquaculture involves the installation of cages directly in the sea. They lack filters or any other method that enables dispersion of particulate wastes. This leads to accumulation of particulate matter in the vicinity of the cages and the organic enrichment of sediments in the surroundings, particularly immediately below the cages.[55] Studies on the dispersion of contaminants from cages are limited and surveillance is focused on the benthic system.[8] Operation of LBMFFs is based on the circulation of seawater through the facilities. The seawater is pumped into ponds and then discharged back into the sea (Fig. 1). Location on highly exposed coasts is often the best way to ensure effluent dilution of intensive LBMFFs, and to avoid input of contaminated water. The strong hydrodynamics lead to rapid dilution and dispersion of contaminants and the toxicant load of the effluents, as well as to the absence of sediment, so that LBMFF outputs mainly affect the nearby water column and living organisms of the surroundings. Culture of fish in tanks is expected to perform a more efficient use of chemical products and feed, so that the toxicant and organic waste load in the effluent should be lower than in caged-based farms. However, tanks require the use of large quantities of biocides (to disinfect facilities) and drugs (to maintain optimum health of the fish).

5. Integrated EMP for LBMFFs

5.1. Definition of the pre-operational state

EMPs often have to be developed for LBMFFs that are already in operation. In such cases, the environmental status of the area before being affected by the activities and the causes of the observed effects must be determined by retrospective techniques. Retrospective techniques involve the analysis of different parameters along an impact gradient to a distance where farm effects are negligible and the parameters cease to vary with the distance. The parameters values may become independent of the distance to the contaminants output, and consequently define the preoperational state. The pre-operational state represents the

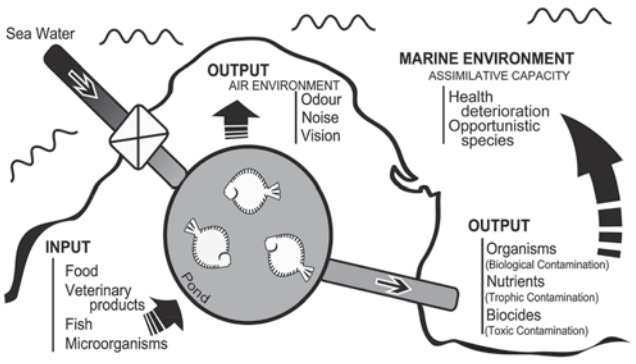


Fig. 1 Diagram of the source of potential impacts of land-based marine fish farms on the marine environment.

reference or control conditions, and can be defined by studying the gradient of both trophic and toxic effects associated with the LBMFF.

However, in the future it is considered optimal for an EMP to be developed, and the pre-operational state be characterized, prior to the beginning of operations of a LBMFF.

5.2. Weight of evidence approach

The application of research based on the weight of evidence (WOE) may offer a comprehensive view of the relationships between the parameters observed in an EMP. The WOE approach categorises parameters in lines of evidence (LOEs), so that each LOE answers fundamental questions such as: what contaminants are present in the environment? Are they bioavailable? Do they have detrimental effects on organisms? Do they involve a risk to the ecosystem? The integration of different LOEs enables identification of current and potential environmental impacts related to a combination of stressors.[56–58] While this approach has primarily been used to assess the quality of sediment,[56–58] it is highly desirable for determining the relationship between the source of contamination and the extent of impact. Before applying this type of approach, it is important to select suitable variables or descriptors (included in each LOE) and their spatio-temporal distribution, to include them on each axis, taking into account the specific nature of each facility and its location.

Here, a triaxial approach comprising three LOEs is proposed for designing an integrated EMP for LBMFFs (Fig. 2). The LOEs are: (1) assessment of contamination exposure, by determining the concentrations of fish farming related contaminants, both in the water column and organisms, and the difference from background values, (2) assessment of effects on biota, both trophic and toxic, *in situ* and in the laboratory, at different levels of biological organization, and (3) assessment of the ecological integrity, by determining possible alterations in the structure and behaviour of local communities. A more detailed description of the proposed EMP is shown in Fig. 3, in which each LOE is defined by the tools used and the parameters analyzed.

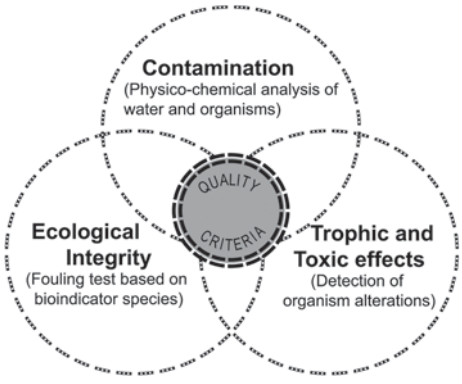


Fig. 2 Summary description of the integrated approach used in this study, consisting of the three selected lines of evidence, in the weight of evidence approach, using the schematic representation of the TRIAD integration.[57]

5.2.1. Contaminant evaluation

5.2.1.1. Physico-chemical analysis. Current regulations for land-based aquaculture propose monitoring a series of physico-chemical parameters in the effluent, to measure the impact of LBMFFs. Environmental quality objectives (EQOs) have already been established in Galicia (NW Spain), the European leader in land-based culture of *Psetta maxima* (approximately 7000 tn per year) (Table 2). EQOs are defined as specific requirements that a body of receiving water must have in order to maintain a high quality of the natural environment. Nevertheless, EQOs are only established for conventional parameters such as pH, conductivity, biological oxygen demand, total nitrogen, nitrites, ammoniacal nitrogen, total phosphates, suspended solids and total organic carbon, while the concentrations of potentially toxic chemicals commonly used in aquaculture (including emerging contaminants) are not taken into consideration. Furthermore, the temporal and spatial frequency of the measures proposed in the current regulations is arbitrary and insufficient for effective environmental monitoring. This shows that even in geographical regions that are pioneering in the aquaculture sector and attempting to be protective of the environment, the formulation and application of regulations for LBMFFs are based on scarce knowledge of the real environmental impact of the activity.

The concentration of nutrients and toxicants associated with fish farming can be measured in the effluents and in environmental matrices, including the water column, interstitial water and sediment. The establishment of background concentrations in the receiving environment enables detection of changes in the chemical composition of the environmental matrices studied and identification of possible sources of contamination. Nevertheless, the influence of natural spatio-temporal variations, especially in the case of nutrients, makes quantification difficult and involves intensive, costly sampling. Furthermore, the low precision of traditional analysis because of the high dilutions of nutrients and the lack of precise models capable of predicting the risk of eutrophication are serious drawbacks for the inclusion of dissolved nutrients in EMPs.[59] For instance, the dilution of ammonia is rapid and measurement of trace elements is difficult, even close to the point of discharge.[60] However, during conditions of high load and low dispersion, classic parameters such as the concentration of ammonia in solution may be appropriate.[61] In the case of toxicants (antibiotics, phytosanitary products, metals and organohalogenated compounds), the analytical procedure is costly and therefore may be considered impractical for monitoring plans.

The extent of contamination derived from aquaculture activities can also be evaluated by measuring the contaminant and nutrient body burdens (bioaccumulation) in indicator or sentinel species,[62] which are ecologically or commercially relevant. Macroalgae and sessile invertebrates that are sufficiently large to facilitate analysis have been found to be suitable bioindicators.[63,64] However, as already mentioned, chemical analyses of nutrients and toxicants may not be appropriate for an EMP owing to the high costs and the methodological requirements for obtaining reliable data. Nonetheless, measurement of chemical body burdens is beneficial, because it provides an indication of bioavailability. Bioavailability studies may contribute to subsequent assessment of the impact of

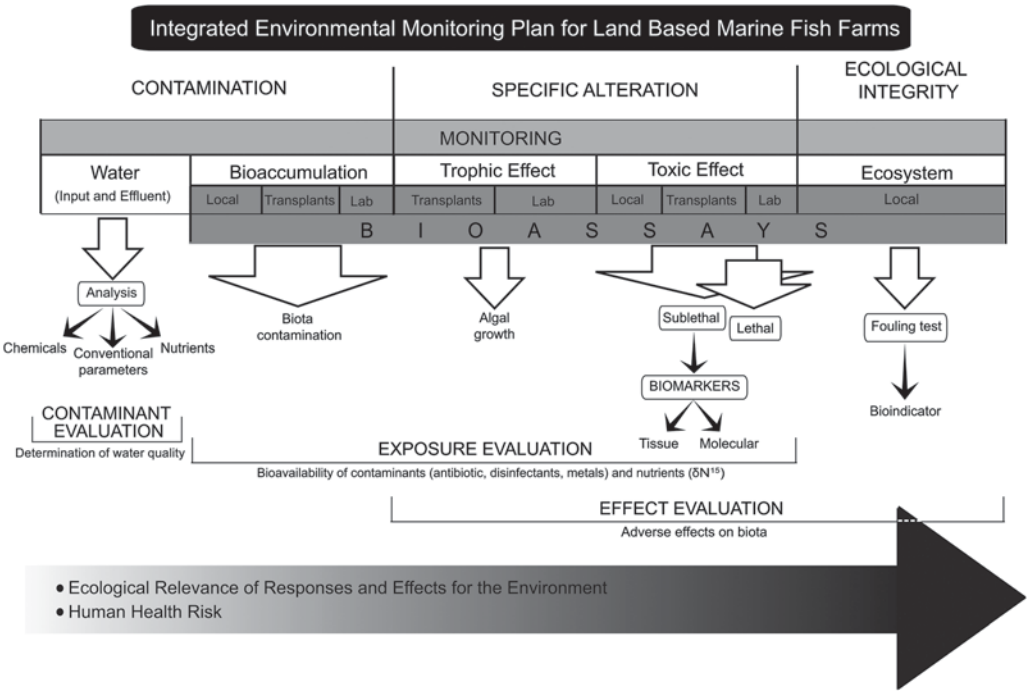


Fig. 3 Diagram showing the Integrated Environmental Monitoring Plan for Land-Based Marine Fish Farms according to the WOE (Weight of Evidence; adapted from ref. 56), which considers three main lines of evidence; chemical analysis, ecotoxicity and ecological integrity.

contamination on organisms and ecosystems, because only chemicals that enter the body of organisms can exert toxic effects,[65] either without previous transformation, *e.g.* metals,[66,67] or after being metabolized, *e.g.* PAHs and pesticides.[66,68] On the contrary, the concentration of nutrients is not necessarily an indicator of ecological risk, in fact, it must be considered as an indicator of exposure. Villares and Carballeira[69,70] found that the nutrient concentration in the water column did not modify the

Table 2 Maximum permitted increases (MPI) and ecological quality objectives (EQO) for monitoring land-based marine fish farm discharges (the case of Galicia, the European leader in land-based culture of *Psetta maxima*) (ref. 120)[a]

Parameter	Number of data	MPI	Median of observed values			EQO
			I	O	O − I	
pH	68	—	8.03 ± 0.03	7.73 ± 0.06	−0.30 ± 0.05	95% cases I − O < 0.30 5% cases I − O < 0.40
Conductivity (μS cm^{-1})	72	—	53 ± 1	70 ± 16	16 ± 17	95% cases O − I < 20 μS cm^{-1} 5% cases O − I < 45 μS cm^{-1}
DBO_5 (mg l^{-1})	59	—	2.93 ± 0.26	6.62 ± 2.42	1.23 ± 0.22	95% cases O − I < 1.5 mg l^{-1} 5% cases O − I < 2.5 mg l^{-1}
N total (mg l^{-1})	157	—	1.34 ± 0.17	1.78 ± 0.24	0.29 ± 0.15	95% cases O − I < 0.3 mg l^{-1} 5% cases O − I < 0.6 mg l^{-1} 100% cases O < 5 mg l^{-1}
N nitrites (mg l^{-1})	167	O − I < 0.05	0.045 ± 0.01	0.075 ± 0.014	0.028 ± 0.01	95% cases O − I < 0.03 mg l^{-1} 5% cases O − I < 0.06 mg l^{-1}
N ammoniacal (mg l^{-1})	54	—	2.33 ± 1.35	2.42 ± 1.56	0.12 ± 0.21	95% cases O − I < 0.15 mg l^{-1} 5% cases O − I < 0.55 mg l^{-1} 100% cases O < 4.5 mg l^{-1}
P total (mg l^{-1})	65	—	0.28 ± 0.05	0.31 ± 0.05	0.01 ± 0.02	95% cases O − I < 0.02 5% cases O − I < 0.05 mg l^{-1} 100% cases O < 0.45 mg l^{-1}
P phosphates (mg l^{-1})	157	O − I < 0.2	0.19 ± 0.04	0.24 ± 0.05	0.05 ± 0.01	95% cases O − I < 0.05 mg l^{-1} 5% cases O − I < 0.15 mg l^{-1} 100% O < 0.35 mg l^{-1}
SS (mg l^{-1})	180	O − I < 5	13 ± 20	13 ± 27	0.68 ± 0.87	95% cases O − I < 1 mg l^{-1} 5% cases O − I < 2.5 mg l^{-1} 100% cases O < 75 mg l^{-1}
TOC (mg l^{-1})	157	O − I < 0.5	6.64 ± 1.72	7.63 ± 1.94	0.90 ± 1.82	95% cases O − I < 1 mg l^{-1} 5% cases O − I < 3.5 mg l^{-1}

[a] I: input water; O: water outlet; O − I: difference.

algal community on the Galician coast (NW Spain), because algae were naturally saturated with nutrients. Therefore, local features must be taken into consideration when evaluating the ecological risk associated with the concentration of dissolved nutrients. In addition, the natural variability and the restrictive thresholds of nutrients for each species, such as the subsistence concentration and the critical concentration, must be defined.[69]

5.2.1.2. $\delta^{15}N$ signal. Anthropogenic sources of N alter the baseline levels of δ^{15}N in marine systems.[71] Thus, the analysis of the δ^{15}N signal can be used to trace and quantify the inputs of organic contamination.[71] The δ^{15}N signal may be interpreted as a measure of nutrient accumulation and as a descriptor of exposure. Nonetheless, we believe that this parameter should be considered separately, due to its multiple advantages in comparison with other chemical analysis.

Measurement of δ^{15}N is simple, rapid and inexpensive. Variations in this parameter in different species have been successfully used to trace nitrogen released from fish farms.[9,71–75] Moreover, the δ^{15}N signal has been shown to be stable and clearly tends to increase with increasing farm waste discharges.[76] Therefore, maps of the distribution of the δ^{15}N signal in biomonitors can be used to distinguish "hot spots" of activity and to verify the effectiveness of environmental protection measures. However, the indicator role of δ^{15}N in multifocal situations of pollution may be questionable. Moreover, N enrichment does not necessarily indicate the influence of anthropogenic discharges.[69,70] *Fucus* spp. (macroalgae) are preferred for δ^{15}N surveys because they are cosmopolitan, abundant and can cover the existing salinity gradients of open coasts and estuaries.[77] Nevertheless, the possible absence of the main biomonitors requires intercalibration of responses with other species so that they can be used as alternative biomonitors. Rey-Asensio *et al.*[76] found no significant differences in δ^{15}N between pairs of macroalgal species collected from the same localities, demonstrating that this species could be used indiscriminately for monitoring purposes. The use of biomonitors requires the definition of reference levels and the natural variability in δ^{15}N.[76]

The δ^{15}N signal has been found to be significantly correlated with parameters indicating trophic and toxic effects, such as algal growth and tissue damage.[78,79] This has opened up a new line of research which examines the suitability of ^{15}N as an indicator of risk associated with discharges from farms, not only as an indicator of exposure to organic contamination. Once the relation between δ^{15}N and effect parameters is well characterized, δ^{15}N analysis may replace other more laborious and expensive analyses.

5.2.2. Specific alterations. *In situ* and laboratory bioassays, together with field surveys on local organisms, allow the study of the effects that the exposure to chemicals (individually or in mixtures), nutrients and contaminated matrices (*e.g.* residual effluents, seawater and sediment) have on organisms, in the short and long term, under natural and controlled conditions.[80] The effects can be analyzed from molecular to community levels and from histological, physiological, behavioural and ecological viewpoints, so that further ecological damage can be assessed.

Selection and/or design of a battery of bioassays and field surveys must be done to achieve a comprehensive evaluation of the potential environmental effects of LBMFFs and to understand the toxicity mechanisms of the substances in farm effluents, individually and in mixtures. In this way, the variability in the effluent flow and contaminant load will be taken into account. On one hand, batteries of bioassays and surveys should include different test species, so that the effects of contaminants on various species are studied, but the technical and methodological problems posed by community tests are avoided.[81] The species selected should represent different taxa, trophic levels and life strategies in order to cover different routes of entry of toxicants and organism sensitivities, and they should be appropriate indicators or sentinel species, *i.e.* they should be ecologically relevant, abundant, easy to obtain, maintain and cultivate, and sensitive to a wide range of pollutants.[82,83] This should provide easily measurable and reliable effects or results, and a simple, standardized, reproducible and cost-effective methodology.

In situ bioassays should counteract deficiencies in laboratory tests in replicating complex environmental conditions and facilitate the prediction of effects in the field. In a monitoring framework, *in situ* bioassays represent an intermediate situation between the experimental control of laboratory assays and the environmental realism of field monitoring (Fig. 4).[84] Local species have traditionally been studied and constitute a powerful tool in environmental monitoring. Nevertheless, in recent years, the advantages of *in situ* bioassays with transplanted organisms have been pointed out,[65,85] mainly because transplanted organisms have been found to be more sensitive than local organisms, which are likely to be adapted to existing conditions.[86]

Transplantation methodologies have been proposed as a valuable tool for further decision making and management, as compared with chemical analysis, laboratory tests and studies on local organisms.[84]

5.2.2.1. Trophic effects. Effluents from LBMFFs may alter the trophic status of the water bodies as a result of the large input of organic matter. Measurement of parameters that enable assessment of nutrient bioavailability and the eutrophication potential in an aquatic system is therefore recommended to indicate the possible ecological changes caused by the LBMFFs.[49] Trophic effects can be determined by laboratory and *in situ* bioassays, and by direct measurements made in the field.

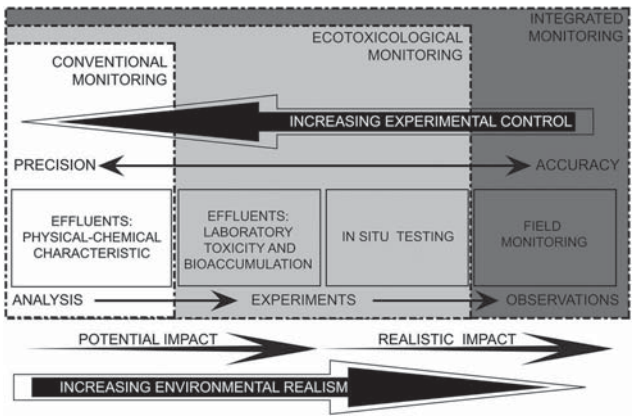


Fig. 4 Diagram illustrating the increase in the environmental realism and accuracy of methods applied in relation to the amount and type of monitoring (modified from Crane *et al.*[84]).

Chlorophyll. The chlorophyll concentration in the water is an indicator of biomass and primary productivity of phytoplankton[87] and at a meso-scale level it may be useful for monitoring harmful algal blooms[49] because these blooms are usually triggered by enrichment of the ecosystem with nutrients (mainly P and N). Nevertheless, chlorophyll measurements for aquaculture monitoring may be limited to local use because of the possible spatio-temporal variability.[86]

Cover by opportunistic macroalgae. The cover or biomass of opportunistic macroalgae species has been proposed as an indicator of eutrophication.[88] Opportunistic macroalgae species with annual life cycles are easily distinguished from perennial species and their presence may directly affect local species.[89] Reference levels of the most abundant perennial macroalgae in the intertidal zone[90] must be known in order to evaluate eutrophication in terms of the disturbance caused by imbalances. Only fixed macroalgae located close to the output must be taken into account since the visual evaluation of green tides caused by high availability of nutrients is not suitable for assessing local impacts, because different physical (temperature, light, hydrodynamics) and biological factors (such as inter-specific competition and grazing) may also determine the abundance of such tides, which differ from one area to another.[91]

Variations in annual primary productivity. The parameter that best characterizes eutrophic processes is the annual primary productivity.[92] However, because of the cost and complexity of *in situ* measurement of macrophytobenthic production, measurement of the biomass at the end of the growing season is often preferred. Moreover, the high renewal rate of phytoplankton biomass requires numerous measurements for accurate estimation of annual production, which reduces the applicability of this method.[93]

Algal growth bioassays. Algal growth bioassays can be carried out with macro- and microalgae, in the laboratory and in the field. Laboratory microalgae tests enable evaluation of the trophic capacity of effluents[94] and calculation of the dilution necessary to prevent significant effects on growth,[95] relative to a control. These tests have also revealed the possible inhibitory and inductory effects of LBMFF discharges on algal growth, since on one hand, nutrients induce growth, and on the other hand, toxic chemicals inhibit growth.[95] Tests of the growth of transplanted macroalgal discs and phytoplankton surveys in the field have been found to be suitable for evaluating the trophic effects of fish farming in the aquatic system.[59,79] Algae exhibited a clear negative gradient in nutrient absorption capacity along with the distance from the farm, and responded rapidly (6–9 days of exposure).

Given the information provided, fertility bioassays and modelling local eutrophication by measuring nutrient bioaccumulation (using *e.g.* $\delta^{15}N$) are recommended as tools for monitoring nutrient enrichment in order to predict environmental degradation at local levels.

5.2.2.2. Toxic effects. Aquaculture requires the use of chemical, man-made products to maintain healthy farm facilities and improve production. Large amounts of these products are released to the environment, where they represent a threat to biota because of their capacity to enter organisms and disrupt the normal functioning of target molecules, causing toxicity, firstly at the cell level, but with potential consequences for organisms, community and ecosystems.[80] Study of the toxicity of emerging man-made contaminants is fundamental for predicting and preventing the impact of aquaculture on the environment.

Bacterial bioassays. Microorganisms are key members of ecosystems, in terms of both biomass and ecological relevance, since they play an essential role in geochemical cycles and energy transformation.[82] Some standardized bacterial tests are already contemplated by the European environmental legislation (EU Water Framework: Directive 2000/60 EC), *e.g.* Microtox®. Such tests respond to a wide range of chemicals and chemical mixtures[96] and have been shown to be suitable for assessing the toxicity of biocides that are used in fish farming, such as antibiotics,[97–100] disinfectants,[101] and excretion products.

Microalgae bioassays. As the basis of the food chain, microalgae play a fundamental role in aquatic ecosystems, so that alterations in microalgal populations may affect higher levels of biological hierarchy and disrupt the ecosystem.[102] The algal growth inhibition test has also been standardized.[103] De Orte *et al.*[95] found that microalgae species were highly responsive to the metabolic waste and chemicals released from turbot LBMFFs, and displayed sensitivities to biocides similar to those displayed by bacteria, and greater than those displayed by fish and invertebrates.

Sea urchin embryo development test. Most studies that monitor the quality of seawater use at least one invertebrate as a test organism. Tests with larvae or embryos of sea urchins, clams and oysters are very sensitive to chemical compounds of different types, and are simple and inexpensive.[81] Tests using sea urchins have been recommended for a wide spectrum of bioassays for the evaluation of marine sediments, according to their applicability, variability, cost-effectiveness, and standardization.[104,105] The suitability of sea urchin embryo development tests to evaluate water quality affected by fish farming in cages and estuaries has been demonstrated.[55,106]

Biochemical biomarkers. Biochemical biomarkers are measurable molecular changes related to the metabolism of xenobiotics, detoxification and toxicity induced by pollutants.[85] They indicate the presence of toxic substances before these substances produce damage at higher levels of biological organization, and can therefore anticipate irreversible environmental damage. Biomarkers can be the endpoint of laboratory and *in situ* bioassays using transplanted organisms, or they can be measured in local organisms. Bivalve molluscs, especially *Mytilus* spp., are proposed as biomonitors for analyzing biomarkers. *Mytilus* spp. are widely distributed and abundant on coasts where LBMFFs are located. The suitability of *Mytilus* spp. for this type of biomonitoring has been demonstrated, and the natural variability of biomarkers has been defined.[107,108]

Histopathological biomarkers. Histopathological examination of fish and invertebrates appears to be a valuable method for

Table 3 Characteristics of the main components of the integrated model aimed at environmental monitoring of land-based marine fish farms[a]

LOE	Component			Methodology	Priority options
Contamination exposure	Fish farm effluent			- Sediment texture - pH - Conductivity - Acid volatile sulfides - Redox potential - Nitrogenous compounds - Phosphates - Suspended solids - Turbidity - Biological oxygen demand - Organic matter - Isotopic relations - Concentration of toxicants: metals, antibiotics, sanitary products, organohalogenated compounds, …	*(a) Effluent parameters* (difference water outlet–input) - pH - Suspended solids - Total organic carbon - NH_4^+ *(b) Macroalgae* - $\delta^{15}N$ - Body burden of contaminants *(c) Invertebrates* - $\delta^{15}N$ - Body burden of contaminants
	Matrix zone* A	Water	Dissolved compounds; particulate matter		
		Sediment	Solid phase; interstitial water		
	Biota zone A	Key species; indicator species; and commercial species			
Specific alteration	Fish farm effluent		Dissolved compounds; particulate matter		*(a) Laboratory bioassays* *Matrix*: effluent, metabolites, and toxicants *Test*: - *Vibrio fisheri* (Microtox) - Microalgal growth - Embryo development (sea urchins and molluscs) *(b) In situ bioassays* *Macroalgae*: - Growth (transplants) *Invertebrates (bivalve transplants)*: - Survival (juveniles) - Biomarkers (adults) *(c) Field surveys* *Phytoplankton*: - Pigments/fluorescence Chl *Invertebrates*: - Deformities/asymmetry - Biomarkers
	Matrix zone A	Water	Dissolved compounds; particulate matter		
		Sediment	Solid phase; liquid phase	Bioassays: - Acute/chronic - Laboratory/*in situ* with transplanted species Field surveys with local species End points: - Fecundity - Larval development - Growth/productivity - Biomarkers - Behaviour/mobility - Survival	
Ecological integrity	Benthos zone A	Natural hard/soft substrate	Epifauna/infauna (macro and meso invertebrates)	Structural parameters of populations and communities: - Presence - Abundance - Dominance - Richness and species diversity - ABC curves - Meta-analysis - Aggregation of opportunistic feeders Functional parameters of populations and communities: - Alteration of assemblages - Pigment composition - Productivity/biomass	Opportunistic and epiphytes (bioindicator species) - Presence - Abundance - Dominance Colonizing community (fouling test) - Productivity/biomass - Ecological profiles: bioindicator species - Specific diversity
		Artificial substrates (fouling)	Flora (diatoms and macroalgae)		
	Plankton zone A–B	Phytoplankton; zooplankton			
	Pelagic zone A–B	Fish			

[a] *Zones are defined by the spatial extent of the impact and on the basis of the residence time of the water mass.[49] A: hours (local), B: days to weeks, C: weeks to months (regional).

in situ assessment of short- and long-term toxic effects.[109,110] The sensitivity of sublethal histological responses enables accurate identification of the real extent of anthropogenic impacts.[111] The study of histological damage in local and transplanted organisms has been shown to be a reliable tool for evaluating the effects of discharges from LBMFFs and the extent of the impact.[78]

5.2.3. Ecological integrity. Ecological integrity can be measured in terms of community structure and functionality, which can be described by macroscopic parameters that enable detection of changes and comparison of scenarios. However, the procedures involved in obtaining these parameters are often complex, costly and slow.

Indices describing the relationships among taxonomic groups of the plankton community can be applied, but these indices show high spatial and temporal variability, so that their use requires frequent and regular monitoring,[112] and they are not particularly useful for monitoring a local scale.

The European legislative approach to managing and protecting aquatic systems, the Water Framework Directive, includes several multi-metric indices to assess the benthic ecological quality of coastal and transitional waters, *e.g.* the benthic ecosystem quality index (BEQI).[113] These indices have shown great consistency with anthropogenic pressure gradients in different aquatic environments, and with each other, and are emerging as valuable methods for evaluation of ecological integrity.[41] Nevertheless, they have been designed for assessment of soft substrates, which are not found in the sites where intensive LBMFFs are usually located. Therefore, surveillance of the ecological integrity of highly exposed coasts should focus on benthic indicators from hard substrates in the intertidal zone.[114]

Pollutants affect photosynthetic electron transport, causing different effects, but always with the same consequence: a change in pigment composition, particularly chlorophyll.[115] Chlorophyll fluorescence has been reported to be a reliable, rapid and economic method for assessing the impact of contamination on microalgal communities[116–118] because it is directly related to biomass and phytoplankton primary productivity.[49]

A valuable technique for monitoring the integrity of hard substrate communities and the extent of the fish farm impact is to study the colonizing capacity of these communities on clean, sterile surfaces (fouling test).[119] This fouling capacity can be measured by placing artificial substrates along a gradient in the zone of influence of discharges. However, the method is laborious and expensive, results are slow to obtain and their analysis is complex and time-consuming. Community analysis should therefore be carried out with simple ecological indices based on detection of bioindicator species (both opportunistic and epiphytic).

6. Conclusions

Land-based marine aquaculture is presented to the public as a clean activity. However, this is far from the truth. LBMFFs release large amounts of organic matter and, to a lesser extent, toxic chemicals to coastal waters. Moreover, effective controls and regulations have not yet been developed. The apparent absence of environmental effects is due to the strong hydrodynamics on the coasts where facilities are located, which provokes rapid dispersion of the contaminants, so that contamination of the input water is avoided. Thus the area where the effects of LBMFF discharges are observable or measurable is not very large. In order to establish the boundaries of this area, the parameters selected for evaluating the impact of LBMFFs should be studied by following a non-linear gradient, starting at the point of output of the LBMFF effluent and following the direction of the prevailing current and by determining the pre-operational state.

A WOE approach is recommended for designing EMPs to include all aspects necessary for a comprehensive understanding of the interactions between the activity and the coastal aquatic system: the concentrations, bioavailability and the environmental effects of the contaminants released. The different LOEs should consider parameters that indicate ecological changes, and not just mere descriptors of the actual conditions. The lack of sediments in the receiving coastal areas has been found to be the main limiting factor in the selection of parameters, since most of the methods used to monitor aquatic systems affected by aquaculture activities focus on sediments. Therefore, specific EMPs for LBMFFs are required owing to the particular characteristics of intensive marine land-based aquaculture and the coastal areas where farms are located.

The extent of contamination can be assessed by determining the concentration of nutrients and chemical products in the effluent and organisms, although this method is not recommended for routine use in EMPs. On the contrary, the $\delta^{15}N$ signal has been found to be a reliable, simple and rapidly measured parameter for evaluating contaminant exposure over a spatial gradient.

Bioassays are essential tools for assessing the real impact of LBMFF, in contrast with conventional physico-chemical parameters. Standard and rapid laboratory tests, complemented with *in situ* bioassays and studies on local organisms, both according to a spatial gradient, are strongly recommended. Trophic effects can be reliably determined by measuring transplanted macro- and microalgal growth. Toxic effects can be evaluated by measuring a wide spectrum of responses at different levels of biological organization, including biochemical and histological changes, physiological alterations, and survival, in species from different trophic levels. A battery of tests including at least one species of bacteria, one species of microalgae and one invertebrate species is recommended.

The study of the ecological integrity is a complex issue and all the available methodologies entail intensive work. Fouling tests and the analysis of community structures, with special attention to bioindicator species, will simplify such study.

The methods proposed here for an EMP for LBMFFs are summarized in Table 3. The EMP could also be adapted to other activities that impact highly exposed coasts, where sediment is scarce.

Acknowledgements

This study was funded by the *Junta Nacional de Cultivos Marinos* (JACUMAR) within the project entitled: "Selection of indicators, determination of reference values, design of programs and protocols and measures for environmental studies in marine aquaculture (INDAQUA)" (2008–10). We are conducting studies and experiments as part of this project, to validate scientific support for the design of an EMP. Carlos Carballeira is supported by a Predoctoral Fellowships Programme at the University of Cadiz (Spain). To Professor Alejo Carballeira Ocaña in commemoration of his 40 years as an environmental pollution researcher. Prof. Carballeira served as my teacher, academic counselor, and role model. I was fortunate enough to have him as my research advisor.

References

1 E. Roque D'Orbcastel, D. Sauzade, G. Ravoux and D. Coves, *Methodological Guide for the Elaboration of Creation of Authorization Classified Installations for the Environment Protection (CIEP) in Marine Fish Culture for the Corsica Area*, IFREMER, Brest, 2004.
2 P. Read and T. Fernandes, *Aquaculture*, 2003, **226**, 139–163.
3 CETGA, *Desarrollo de un método para minimizar los residuos de los efluentes de plantas acuícolas y su posible valorización*, Centro Tecnológico Gallego de Acuicultura, Aguiño, Galicia, 2005, p. 62.

4 R. Inokuchi, K. I. Kuma, T. Miyata and M. Okada, *Physiol. Plant.*, 2002, **116**, 1–11.
5 J. A. Camargo and A. Alonso, *Environ. Int.*, 2006, **32**, 831–849.
6 J. S. Gray, R. S.-s. Wu and Y. Y. Or, *Mar. Ecol.: Prog. Ser.*, 2002, **238**, 249–279.
7 T. Källqvist and A. Svenson, *Water Res.*, 2003, **37**, 477–484.
8 A. Modica, D. Scilipoti, R. La Torre, A. Manganaro and G. Sarà, *Estuarine, Coastal Shelf Sci.*, 2006, **66**, 177–184.
9 G. Sarà, D. Scilipoti, A. Mazzola and A. Modica, *Aquaculture*, 2004, **234**, 199–213.
10 G. Sarà, *Mar. Environ. Res.*, 2007, **63**, 390–408.
11 N. Bandow, R. Altenburger, U. Lübcke-von Varel, A. Paschke, G. Streck and W. Brack, *Environ. Sci. Technol.*, 2009, **43**, 3891–3896.
12 Y. Tal, H. J. Schreier, K. R. Sowers, J. D. Stubblefield, A. R. Place and Y. Zohar, *Aquaculture*, 2009, **286**, 28–35.
13 D. Cavallo, A. Pusceddu, R. Danovaro and A. Giangrande, *Mar. Pollut. Bull.*, 2007, **54**, 622–625.
14 G. K. Reid, M. Liutkus, A. Bennett, S. M. C. Robinson, B. MacDonald and F. Page, *Aquaculture*, 2010, **299**, 165–169.
15 A. Neori, *J. Appl. Phycol.*, 2008, **20**, 567–570.
16 A. Tello, R. A. Corner and T. C. Telfer, *Environ. Pollut.*, 2010, **158**, 1147–1158.
17 J. Arthur, C. Lavilla-Pitogo and R. Subasinghe, *Use of Chemicals in Aquaculture in Asia*, Southeast Asian Fisheries Development Center (SEAFDEC), Tigbauan, 1996.
18 F. C. Cabello, *Environ. Microbiol.*, 2006, **8**, 1137–1144.
19 T. Matsumoto, M. Kobayashi, T. Moriwaki, S. I. Kawai and S. Watabe, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.*, 2004, **139**, 147–152.
20 M. Blázquez, P. T. Bosma, E. J. Fraser, K. J. W. Van Look and V. L. Trudeau, *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol.*, 1998, **119**, 345–364.
21 D. G. Douet, H. Le Bris and E. Giraud, *Options Méditerranéennes*, 2009, **86**, 105–126.
22 R. Rodil, A. M. Carro, R. A. Lorenzo and R. Cela, *Chemosphere*, 2007, **67**, 1453–1462.
23 A. Ikem and J. Egilla, *Food Chem.*, 2008, **110**, 301–309.
24 D. J. Woods and C. S. Knauer, *Int. J. Parasitol.*, 2010, **40**, 1177–1181.
25 K. Kummerer, *J. Antimicrob. Chemother.*, 2003, **52**, 5–7.
26 J. M. Rose, R. J. Gast, A. Bogomolni, J. C. Ellis, B. J. Lentell, K. Touhey and M. Moore, *FEMS Microbiol. Ecol.*, 2009, **67**, 421–431.
27 A. K. Sarmah, M. T. Meyer and A. B. A. Boxall, *Chemosphere*, 2006, **65**, 725–759.
28 M. Tamminen, A. Karkman, J. Corander, L. Paulin and M. Virta, *Aquaculture*, 2011, **313**, 15–23.
29 L. Burridge, J. S. Weis, F. Cabello, J. Pizarro and K. Bostick, *Aquaculture*, 2010, **306**, 7–23.
30 C. B. Veatch, *White's Handbook of Chlorination and Alternative Disinfectants*, John Wiley & Sons, New Jersey, 5th edn, 2010.
31 S. J. Rowland, M. Nixon, M. Landos, C. Mifsud, P. Read and P. Boyd, *Aquacult. Res.*, 2006, **37**, 869–876.
32 T. Tišler and J. Zagorc-Končan, *Water, Air, Soil Pollut.*, 1997, **97**, 315–322.
33 X. Tang, Y. Bai, A. Duong, M. T. Smith, L. Li and L. Zhang, *Environ. Int.*, 2009, **35**, 1210–1224.
34 H. Ahyayauch, M. Bennouna, A. Alonso and F. M. Goní, *Langmuir*, 2010, **26**, 7307–7313.
35 M. Panouillères, C. Boillot and Y. Perrodin, *Ecotoxicology*, 2007, **16**, 327–340.
36 E. D. Goldberg, *Environ. Monit. Assess.*, 1986, **7**, 91–103.
37 J. Mork, *Aquaculture*, 1991, **98**, 267–276.
38 D. W. Cole, R. Cole, S. J. Gaydos, J. Gray, G. Hyland, M. L. Jacques, N. Powell-Dunford, C. Sawhney and W. W. Au, *Int. J. Hyg. Environ. Health*, 2009, **212**, 369–377.
39 GESAMP, *Revised GESAMP Hazard Evaluation Procedure for Chemical Substances Carried by Ships*, IMO, London, 2002.
40 ICES, *Code of Practice on the Introduction and Transfers of Marine Organisms*, The International Council for the Exploration of the Sea Cesenatico, 2004.
41 Á. Borja, *Bol. Inst. Esp. Oceanogr.*, 2002, **18**, 41–49.
42 A. J. Underwood, *Experiments in Ecology. Their Logical and Interpretation Using Analysis of Variance*, Press Syndicate of the University of Cambridge, Cambridge, 8th edn, 1997.
43 N. J. Hardman-Mountford, J. I. Allen, M. T. Frost, S. J. Hawkins, M. A. Kendall, N. Mieszkowska, K. A. Richardson and P. J. Somerfield, *Mar. Pollut. Bull.*, 2005, **50**, 1463–1471.
44 C. Sanz-Lázaro, M. D. Belando, F. Navarrete-Mier and A. Marín, *Estuarine, Coastal Shelf Sci.*, 2011, **71**, 22–30.
45 R. H. Piedrahita, *Aquaculture*, 2003, **226**, 35–44.
46 D. Serpa and P. Duarte, in *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, ed. R. Russo, Global Science Books, Alabama, 2008, vol. 2, pp. 1–20.
47 M. J. S. Gondwe, S. J. Guildford and R. E. Hecky, *J. Great Lakes Res.*, 2011, **37**, 102–113.
48 C. J. Cromey, T. D. Nickell and K. D. Black, *Aquaculture*, 2002, **214**, 211–239.
49 P. Tett, R. Gowen, D. Mills, T. Fernandes, L. Gilpin, M. Huxham, K. Kennington, P. Read, M. Service, M. Wilkinson and S. Malcolm, *Mar. Pollut. Bull.*, 2007, **55**, 282–297.
50 A. Ervik, P. K. Hansen, J. Aure, A. Stigebrandt, P. Johannessen and T. Jahnsen, *Aquaculture*, 1997, **158**, 85–94.
51 T. F. Fernandes, K. L. Miller and P. A. Read, *J. Appl. Ichthyol.*, 2000, **16**, 138–143.
52 GESAMP, *Monitoring the Ecological Effects of Coastal Aquaculture Wastes*, FAO, Rome, 1996.
53 SEPA, *Regulation and Monitoring of Marine Cage Fish Farming in Scotland. A Procedures Manual Version 1.0*, Scottish Environment Protection Agency, Stirling, 1999.
54 A. Stigebrandt, J. Aure, A. Ervik and P. Kupka, *Aquaculture*, 2004, **234**, 239–261.
55 A. Marin, S. Montoya, R. Vita, L. Marín-Guirao, J. Lloret and F. Aguado, *Aquaculture*, 2007, **271**, 286–297.
56 P. M. Chapman, *Environ. Int.*, 2007, **33**, 492–501.
57 T. Á. DelValls, *Diseño y aplicación de modelos integrados de evaluación de la contaminación y sus efectos sobre los sistemas marinos y litorales y la salud humana*, Ministerio de la Presidencia, Centro de Publicaciones, Madrid, 2007.
58 C. Morales-Caselles, I. Riba and T. Á. DelValls, *Mar. Environ. Res.*, 2009, **67**, 31–37.
59 T. Dalsgaard and D. Krause-Jensen, *Aquaculture*, 2006, **256**, 302–310.
60 C. Y. Cho, J. D. Hynes, K. R. Wood and H. K. Yoshida, *Aquaculture*, 1994, **124**, 293–305.
61 S. Porrello, M. Lenzi, P. Tomassetti, E. Persia, M. G. Finoia and I. Mercatali, *Reduction of Aquaculture Wastewater Eutrophication by Phytotreatment Ponds System. II. Nitrogen and Phosphorus Content in Macroalgae and Sediment*, Elsevier, Amsterdam, 2003.
62 K. Grigorakis and G. Rigos, *Chemosphere*, 2011, **85**, 899–919.
63 P. A. Reis, M. A. Salgado and V. Vasconcelos, *Estuarine, Coastal Shelf Sci.*, 2011, **93**, 269–278.
64 I. Akcali and F. Kucuksezgin, *Mar. Pollut. Bull.*, 2011, **62**, 637–645.
65 J. Ramos-Gómez, M. Martins, J. Raimundo, C. Vale, M. Laura Martín-Díaz and T. Ángel DelValls, *Mar. Pollut. Bull.*, 2011, **62**, 1538–1549.
66 *Bioaccumulation in Marine Organisms: Effect of Contaminants from Oil Well Produced Water*, ed. J. M. Neff, Elsevier Science, 1st edn, 2002.
67 P. S. Rainbow, *Environ. Pollut.*, 2003, **120**, 497–507.
68 P. E. Levi and E. Hodgson, *Toxicol. Lett.*, 1985, **24**, 221–228.
69 R. Villares and A. Carballeira, *Sci. Mar.*, 2006, **70**, 89–97.
70 R. Villares and A. Carballeira, *Mar. Ecol.*, 2004, **25**, 225–243.
71 S. D. Costanzo, M. J. O'Donohue and W. C. Dennison, *Mar. Pollut. Bull.*, 2004, **48**, 514–525.
72 S. Lojen, E. Spanier, A. Tsemel, T. Katz, N. Eden and D. Angel, *Mar. Biol.*, 2005, **148**, 87–96.
73 G. Sará, *Mar. Ecol.: Prog. Ser.*, 2006, **328**, 65–73.
74 T. Dolenec, S. Lojen, G. Kniewald, M. Dolenec and N. Rogan, *Aquaculture*, 2007, **262**, 237–249.
75 D. T. Lin and P. Fong, *Mar. Pollut. Bull.*, 2008, **56**, 245–249.
76 A. Rey-Asensio, I. Viana, C. Carballeira and A. Carballeira, *XVII Simposio Ibérico de Botánica Criptogámica, Tomar*, 2009.
77 J. Aboal and A. Carballeira, *Bancos de Especimenes Ambientales. Una propuesta para Galicia*, Servicio de publicacións e Intercambio científico, Santiago de compostela, 2000.
78 C. Carballeira, J. Espinosa and A. Carballeira, *Mar. Pollut. Bull.*, 2011, **62**, 2633–2641.
79 C. Carballeira, I. Viana, A. Rey-Asensio and A. Carballeira, *XIII Foro dos Recursos Mariños e da Acuicultura das Rías Galegas, O Grove*, 2010.

80 C. Carballeira, M. R. De Orte, I. G. Viana and A. Carballeira, *Ecotoxicol. Environ. Saf.*, 2012, **78**, 148–151.
81 G. Persoone, C. R. Janssen and W. De Coen, *New Microbiotests for Routine Toxicity Screening and Biomonitoring*, Kluwer Academic/Plenum Publishers, New York, 2000.
82 R. N. Coleman and A. A. Qureshi, *Bull. Environ. Contam. Toxicol.*, 1985, **35**, 443–451.
83 C. Peters, S. Becker, U. Noack, S. Pfitzner, W. Bülow, K. Barz, W. Ahlf and R. Berghahn, *Ecotoxicology*, 2002, **11**, 379–383.
84 M. Crane, G. A. Burton, J. M. Culp, M. S. Greenberg, K. R. Munkittrick, R. Ribeiro, M. H. Salazar and S. D. St-Jean, *Integr. Environ. Assess. Manage.*, 2007, **3**, 234–245.
85 M. L. Martín-Díaz, *Determinación de la calidad ambiental de sistemas litorales y de estuario de la Península ibérica utilizando ensayos de campo y laboratorio*, University of Cadiz, 2004.
86 I. Lopes, D. J. Baird and R. Ribeiro, *Chemosphere*, 2005, **61**, 1189–1197.
87 J. L. Iriarte, H. E. González, K. K. Liu, C. Rivas and C. Valenzuela, *Estuarine, Coastal Shelf Sci.*, 2007, **74**, 471–480.
88 R. A. Cohen and P. Fong, *J. Phycol.*, 2005, **41**, 287–293.
89 C. Olabarria, I. F. Rodil, M. Incera and J. S. Troncoso, *Mar. Environ. Res.*, 2009, **67**, 153–158.
90 J. Cremades, I. Bárbara and A. J. Veiga, *Thalassas*, 2004, **20**, 69–80.
91 X. Puente, R. Villares, E. Carral and A. Carballeira, *Actes du 1er Colloque Interceltique d'Hydrologie et de Gestion des Eaux, Rennes*, 1996.
92 V. H. Smith, *J. Plankton Res.*, 2007, **29**, 1–6.
93 D. S. Grundle, D. A. Timothy and D. E. Varela, *Cont. Shelf Res.*, 2009, **29**, 2257–2269.
94 G. Chiaudani and M. Vighi, *Water Res.*, 1974, **8**, 1063–1069.
95 M. De Orte, C. Carballeira and A. Carballeira, *XVII Simposium Ibérico de Botánica Criptogámica, Tomar*, 2009.
96 T. Backhaus, M. Scholze and L. H. Grimme, *Aquat. Toxicol.*, 2000, **49**, 49–61.
97 G. M. Lalumera, D. Calamari, P. Galli, S. Castiglioni, G. Crosa and R. Fanelli, *Chemosphere*, 2004, **54**, 661–668.
98 M. Isidori, M. Lavorgna, A. Nardelli, L. Pascarella and A. Parrella, *Sci. TotalEnviron.*, 2005, **346**, 87–98.
99 M. D. Hernando, S. De Vettori, M. J. Martínez Bueno and A. R. Fernández-Alba, *Chemosphere*, 2007, **68**, 724–730.
100 S. Park and K. Choi, *Ecotoxicology*, 2008, **17**, 526–538.
101 R. Chénier, *Hum. Ecol. Risk Assess.*, 2003, **9**, 483–509.
102 M. Rouco, *Mecanismos genéticos y estrategias adaptativas de productores primarios (microalgas y cianobacterias) en un escenario de cambio global*, Universidad Complutense de Madrid, 2011.
103 OECD, *OECD Guidelines for the Testing of Chemicals*, Organisation for Economic Co-operation and Development, Paris, 2006.
104 M. Nendza, *Chemosphere*, 2002, **48**, 865–883.
105 C. Carballeira, L. Martín-Díaz and T. A. DelValls, *Mar. Environ. Res.*, 2011, **72**, 196–203.
106 C. Carballeira, M. L. Martín-Díaz and T. A. DelValls, 2nd International Conference on Environmental Management, Engineering, Planning and Economics (CEMEPE 09) & Secotox Conference, Mykonos, 2009.
107 R. Bocchetti, C. V. Lamberti, B. Pisanelli, E. M. Razzetti, C. Maggi, B. Catalano, G. Sesta, G. Martuccio, M. Gabellini and F. Regoli, *Mar. Environ. Res.*, 2008, **66**, 24–26.
108 R. Bocchetti, D. Fattorini, B. Pisanelli, S. Macchia, L. Oliviero, F. Pilato, D. Pellegrini and F. Regoli, *Aquat. Toxicol.*, 2008, **89**, 257–266.
109 J. F. McCarthy and L. R. Shugart, *Biomarkers of Environmental Contamination*, Lewis Publishers, California, 1990.
110 R. D. Handy, T. Runnalls and P. M. Russell, *Ecotoxicology*, 2002, **11**, 467–479.
111 I. Riba, M. González de Canales, J. M. Forja and T. A. DelValls, *Mar. Pollut. Bull.*, 2004, **48**, 153–163.
112 J. R. Karr, P. R. Yant, K. D. Fausch and I. J. Schlosser, *Trans. Am. Fish. Soc.*, 1987, **116**, 1–11.
113 K. Buonasera, M. Lambreva, G. Rea, E. Touloupakis and M. Giardi, *Anal. Bioanal. Chem.*, 2011, **401**, 1139–1151.
114 NIOO, *BEQUI*, http://www.beqi.eu/index.php, accessed 16 December, 2010.
115 J. A. Juanes, X. Guinda, A. Puente and J. A. Revilla, *Ecol. Indic.*, 2008, **8**, 351–359.
116 P. B. Fai, A. Grant and B. Reid, *Environ. Toxicol. Chem.*, 2007, **26**, 1520–1531.
117 S. Sánchez-Fortún, F. Marvá, A. D'ors and E. Costas, *Ecotoxicology*, 2008, **17**, 229–234.
118 M. Schmitt-Jansen and R. Altenburger, *Aquat. Toxicol.*, 2008, **86**, 49–58.
119 E. J. Cook, K. D. Black, M. D. J. Sayer, C. J. Cromey, D. L. Angel, E. Spanier, A. Tsemel, T. Katz, N. Eden, I. Karakassis, M. Tsapakis, E. T. Apostolaki and A. Malej, *ICES Journal of Marine Science*, 2006, **63**, 637–649.
120 APROMAR, La acuicultura marina en España, www.apromar.es/Informes, accessed 21 November, 2011.

Anexo II

Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioacumulación de microcontaminantes

Rey-Asensio[1] A., Carballeira[2] C., Viana[1] I.G., Carballeira[1] A.

[1]Grupo de Ecotoxicología, Área de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, España. ana.rey@usc.es
[2]Grupo de Ecotoxicología Marina, Dptº. Química Física, Instituto de Ciencias Marinas de Andalucía, Consejo superior de Investigaciones Científicas, Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, España.

Resumen

La mayoría de los estudios sobre el efecto contaminante de las piscifactorías en jaulas flotantes se refieren a los análisis de los contaminantes que se acumulan en los sedimentos. Como las piscifactorías marinas instaladas en tierra (LBFF) se localizan habitualmente en zonas con un alto hidrodinamismo, los contaminantes emitidos se dispersan fuertemente por la corriente y difícilmente pueden ser analizados en los sedimentos circundantes al ser poco estables y no poseer una fracción fina capaz de acumularlos. Por otro lado, la alta variabilidad temporal y el alto grado de dilución de los contaminantes traza en los vertidos, encarece y dificulta su detección con métodos convencionales. Por ello, el objetivo de este estudio fue seleccionar el mejor biomonitor de los microcontaminantes ligados a las LBFF, para facilitar los protocolos de vigilancia ambiental. El estudio consistió en la recolección de macroalgas, mejillones y anémonas nativas y en la exposición de trasplantes de *Sacharina sacharina* en el área de influencia de diferentes LBFF. Se analizó la acumulación metálica en biomonitores nativos y, por su interés comercial, se determinaron antibióticos y pesticidas en trasplantes de *S. sacharina*. Paralelamente en todos los biomonitores se determinó la señal isotópica δN^{15} como un descriptor de exposición a los efluentes.

Entre los metales determinados no se encontró ninguno que pudiera considerarse un marcador del vertido de las LBFF claro y común a todos los biomonitores. Solamente la

Anemonia sulcata es capaz de bioacumular algunos de los metales analizados de manera significativa. Se observó un incremento del contenido en antibióticos en los transplantes de *sacarina*, aunque los valores cuantificados están por debajo del límite legal para el consumo humano.

Los resultados confirman que la señal isotópica δN^{15} determinada en todos los organismos –especialmente en macroalgas- es un buen descriptor de exposición a los efluentes, integrando la carga contaminante y la capacidad dispersiva del medio.

Palabras clave

Metales pesados, Antibióticos, $\delta^{15}N$, *Fucus vesiculosus*, *F. spiralis* L., *F.serratus Codium tomentosum*, *Anemone sulcata*, *Mytilus galloprovincialis*.

Introducción

Cada día la piscicultura marina se hace mas intensiva debido a la introducción de nuevas tecnologías, el aumento de superficie disponible, las mejoras en la tecnología alimentaria, el aumento del conocimiento de la biología de las especies cultivadas, la mejora de la calidad del agua dentro de las granjas y el incremento de la demanda de productos de la pesca (Read *et* Fernandes, 2003). En el caso de Galicia (NW Spain), su Plan Acuícola contempla ocupar unos 3.10^6 m^2 en granjas marinas instaladas en tierra (LBFF) para llegar a producir 30.000 $t/año^{-1}$ de peces planos, principalmente rodaballo.

Este desarrollo intensivo ha venido acompañado de un incremento del impacto ambiental derivado de estas industrias (Ervik *et al.,* 1997; Fernandes *et al.,* 2002). Entre los impactos contemplados, la eutrofización es un proceso clave debido a los altos niveles de N (amonio, nitritos y nitratos) que son liberados por esta actividad a las comunidades costeras, a menudo limitadas por este nutriente (Cloern, 2001). Otra fuente de posibles impactos está relacionada con el uso de compuestos químicos utilizados en el proceso productivo, fundamentalmente antibióticos, desinfectantes y antifouling, que pueden ser bioacumulados por la biota.

En la actualidad la vigilancia ambiental de las LBFF se basa en el control de vertidos con técnicas de análisis convencionales (pH, O_2, SS, TOC, fosfatos, nitratos, nitritos y amonio). Esta metodología tiene es poco distintiva de los posibles daños que pueden ejercer sobre el ecosistema, puesto que el impacto ecológico de un vertido depende de su carga, de las características (hidrodinamismo, batimetría y geografía) del medio y de la biota receptora (Carballeira *et al.*, enviado). Además, cuando analizamos los resultados de los controles rutinarios realizados (datos suministrados por Augas de Galicia y por diferentes

LBFF) observamos que el alto grado de dilución dificulta la observación de diferencias en las concentraciones de los macrocontaminantes entre el agua de entrada y la de salida de la LBFF. La situación es mucho mas grave cuando se tratan de contaminantes traza (i.e. metales, PAH, biocidas).

Por todo ello, el objetivo de este estudio consiste en buscar posibles biomonitores de microcontaminantes (i.e. metales y metaloides, pesticidas y antibióticos) que puedan ser incluidos en protocolos eficaces de vigilancia ambiental de las LBFF. Para ello los biomonitores, en primer lugar, deberían de ser capaces de informar sobre la inmisión y toxicidad potencial de los microcontaminantes mediante su bioacumulación y, en segundo lugar, que a través de las concentraciones acumuladas se pueda caracterizar la intensidad y extensión del área de influencia de los vertidos.

Materiales y métodos

Área de Estudio

Para la realización del estudio se seleccionaron ocho LBFF situadas en zonas de la costa gallega con distintas características hidrodinámicas (Fig. 1) sin ningún foco de contaminación significativo en su entorno. Las piscifactorías seleccionadas están centradas en el cultivo de rodaballo (*Psetta maxima, Linnaeus*, 1758) y realizan sus vertidos al medio marino sin tratamiento previo. La producción anual de rodaballo adulto de las granjas seleccionadas varía de 300 t/año (EE5) a 2250 t/año (EE1). Todas las granjas presentan un sistema de circulación abierto, en las que el caudal de agua de salida varía entre 5 m^3/s (EE2) y 11 m^3/s (EE1).

Localización de la estaciones de muestreo (EM)

En cada una de las granjas se diseñó un gradiente ambiental en la dirección predominante de la corriente desde la salida del vertido (Fig.2). En la Tabla I se recogen las estaciones de muestreo (EM) seleccionadas para cada biomonitor y su distancia al vertido. El muestreo de macroalgas y anémonas nativas en cada EM se realizó en la zona intermareal. Los transplantes de sacarina se realizaron sobre un cabo sembrado (1m) suspendido en el mar de una boya.

Los muestreos se realizaron en la época de máxima producción en julio 2008 y los transplantes se mantuvieron desde noviembre (2008) a febrero (2009).

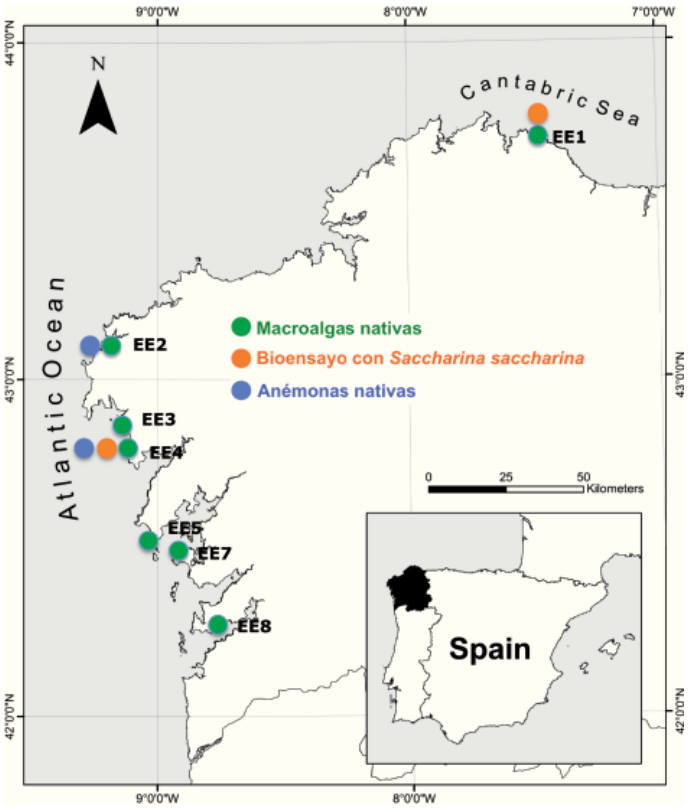


Figura 1.- Localización en el litoral de Galicia (NW España) de las piscifactorías marinas instaladas en tierra (LBFF). Los círculos indican las LBFF que fueron utilizadas para cada biomonitor: macroalgas (verde); anémonas (azul); transplantes de laminaria (naranja); los mejillones fueron muestreados en las 7 LBFF.

Figura 2.- Ejemplo de localización de las estaciones de muestro (EM) localizadas a modo de gradiente en una LBFF tipo (EE4) para diferentes biomonitores utilizados en el estudio.

Tabla I.- Localización en gradiente de las estaciones de muestreo en cada piscifactoría (EE) según el biomonitor utilizado.

Especie	EE	Distancia al vertido (m)						
		0	50	100	200	400	800	1000
Fucus vesiculosus	EE1							
	EE2							
	EE3			x	x		x	
	EE4	x	x	x	x	x	x	x
	EE5		x	x	x		x	
	EE7		x	x			x	
	EE8		x	x	x		x	
	EE1		x	x	x	x	x	
	EE2		x	x			x	x
	EE3		x	x			x	
	EE4							
	EE5							
	EE7		x	x			x	
	EE8		x	x				
Mytillus galloprovincialis	EE1							
	EE2	x		x		x		
	EE3	x		x		x		
	EE4	x				x		
	EE5	x			x			
	EE7	x			x			
	EE8	x		x				
Sacharina saccharina	EE1		x	x	x	x	x	
	EE4		x	x	x	x	x	
Anémone sulcata	EE2	x		x				x
	EE4	x	x	x		x		x

Biomonitores

- **Macroalgas nativas**

Las macroalgas reúnen las características principales para ser utilizadas como biomonitores, ya que son sedentarias, tienen una amplia distribución geográfica y son fáciles de muestrear e identificar (Phillips, 1977). La macroalga seleccionada como biomonitor principal (Viana *et al.,* 2011) fue *Fucus vesiculosus* (L.), pero como en el entorno de las LBFF no siempre fue posible encontrarlo se seleccionaron como biomonitores secundarios otras especies ubiquistas en la costa de Galicia como *F. spiralis* L. y *F.serratus* L. En el caso de que no hubiera ninguna fucácea se optó por muestrear el alga verde *Codium tomentosum*

(Hudson) Stackh., muy frecuente en las inmediaciones de las piscifactorías.

El muestreo se llevó a cabo en la franja mesolitoral con marea baja. Cada EM abarca una franja de 50 metros de línea de costa; en cada una de ellas se recogieron de forma homogénea más de 30 ejemplares fijados al sustrato, siguiendo una línea en zigzag en la franja litoral donde crecen las algas, con el fin de disminuir lo máximo posible el grado de variabilidad interindividual. Estos ejemplares se juntaron en una muestra compuesta por cada EM. Las algas fueron lavadas *in situ* con agua de mar y se transportaron refrigeradas (4ºC) al laboratorio, donde fueron conservadas a –30ºC hasta su procesado.

Antes de procesar el material se descongeló a temperatura ambiente y se lavó cuidadosamente con abundante agua bidestilada con el fin de eliminar los restos de sedimentos y de organismos epifitos; las partes viejas o dañadas fueron eliminadas. Para los análisis se extrajeron, con una espátula de vidrio, los 3 cm distales de los talos vegetativos, homogeneizándose finalmente la muestra húmeda con un triturador de laboratorio (Waring Blender 34BL99). El material fue secado a 45ºC en estufa de tiro forzado, antes de ser pulverizado con un molino ultracentrífugo (Retsch ZM 100), con el fin de que el 100% del tamaño de las partículas fuera inferior a 120µm (al menos el 80% es <50 µm).

- **Bioensayo de macroalgas**

Se escogió la macroalga *Saccharina saccharina* L. para la realización de los transplantes, por ser una especie autóctona con un uso comercial cada vez más extendido. Este bioensayo se realizó en EE1 y EE4.

Se obtuvieron individuos de dicha especie cultivados y suministrados por el Centro Oceanográfico de Santander. En cada boya (EM) se sujetó un cabo de un metro de longitud desde la superficie del agua y en sentido longitudinal al cabo principal. En cada cabo había entrelazados unos 100-200 individuos. Fueron expuestos al vertido 77 días hasta el momento de la recolección.

Tras el periodo de exposición para determinar los compuestos bioacumulados ($\delta^{15}N$, metales y metaloides, antibióticos) de cada EM y recoger la variabilidad existente se obtuvo una muestra compuesta formada por tres especimenes de diferente longitud. Los ejemplares fueron lavados *in situ* con agua de mar y se transportaron refrigerados (4º C) al laboratorio. En el laboratorio el material fue lavado de nuevo con agua bidestilada con el fin de eliminar los restos de sedimentos y organismos epifitos, y las partes viejas o dañadas fueron eliminadas. Las muestras se homogeneizaron con un triturador de laboratorio (Waring Blender 34BL99) y se secaron a 45ºC en estufa de tiro forzado antes de ser homogeneizadas con un molino ultracentrífugo (Retsch ZM 100).

- **Anémonas nativas**

El muestreo de *Anemonia sulcata* L. se realizó en la zona intermareal, tomando 30 individuos de charcas diferentes con el fin de representar la variabilidad existente en cada EM. La muestra compuesta de cada EM se traslada al laboratorio en vivo y se eliminan los restos de arena y partículas en detalle de cada individuo. Se secan con papel de filtro y se homogeniza la muestra en un triturador de laboratorio (Waring Blender 34BL99). La papilla resultante se congeló a -80ºC y posteriormente se liofilizó. El material resultante fue pulverizado con un molino ultracentrífugo (200 µm) (Retsch ZM 100).

- **Mejillón nativo**

El muestreo se realizó en el entorno de 7 piscifactorías donde se recogió *M. galloprovincialis* L. nativo, de la región intermareal, en EM localizadas en forma de gradiente no lineal desde el emisario. Se establecieron de 3 a 5 EM dependiendo de la existencia de ejemplares y de la morfología litoral en el entorno de las LBFF. En cada EM se tomaron mas 10 mejillones (4-5 cm de longitud de la valva), distribuidos homogéneamente en un área de aproximadamente 15 metros de diámetro. Los bivalvos son trasladados en frío al laboratorio, y una vez extraída la vianda, se homogeneiza la muestra con un triturador de laboratorio (Waring Blender 34BL99). El material fue secado a 45º C en estufa de tiro forzado, antes de ser pulverizado con un molino ultracentrífugo (Retsch ZM 100)

Determinación analítica

- **Determinación de isótopos estables de N ($\delta^{15}N$)**

Una alícuota (3 mg) de la muestra homogeneizada de cada EM se empaquetó en cápsulas de estaño (EuroVector tin capsules). Las cápsulas se almacenaron en un desecador hasta su análisis en la Unidad de Técnicas Instrumentales de Análisis (UTIA) de los Servicios de Apoyo a la Investigación de la Universidad de A Coruña. Las muestras se sometieron a combustión en un analizador elemental FlashEA1112 (ThermoFinnigan) unido a un espectrómetro de masas de relaciones isotópicas Delta^{plus} (ThermoFinnigan). La acetanilida fue el patrón empleado para la cuantificación del contenido de nitrógeno. La calibración del gas de referencia para ^{15}N atmosférico se realizó con los patrones IAEA-N-1 ($(NH_4)_2SO_4$), IAEA-N-2 ($(NH_4)_2SO_4$) y IAEA-NO-3 (KNO_3). Las relaciones isotópicas ($^{15}N/^{14}N$) de las muestras se comparan con el estandar (N_2 atmosférico) para hacer las proporciones obtenidas en las distintas muestras comparables. La cantidad relativa de ^{15}N en la muestra ($\delta^{15}N$) se determinó mediante la fórmula: $\delta^{15}N$ (‰) = $[(R_{sample}/R_{standard}) -1]*10^3$, donde R es la relación $^{15}N/^{14}N$. El error global, determinado mediante la utilización de réplicas analíticas (n=23) fue 2%.

- **Determinación de metales y metaloides**

La determinación de las concentraciones metálicas se realizó sobre la muestra

compuesta seca y homogeneizada de cada EM. La extracción de la algas fue llevada a cabo en bombas de Teflón® (Advanced Composite Vessels) en un horno microondas (CEM MDS 2100), añadiendo 10 mL de HNO_3 (65%) a 0.5 g de peso seco de muestra. Para las anémonas y los mejillones la digestión se realizó en dos etapas, una primera con ácido nítrico y la segunda con peróxido de hidrógeno. Cada nueve muestras se incluyó: un blanco, una replica analítica al azar y materiales de referencia certificados BCR (Community Bureau of Reference), con el fin de determinar la eficiencia de la digestión.

En primer lugar, se realizó un análisis cualitativo de las muestras mediante ICP-AES en el Departamento de Química Analítica (USC). Posteriormente se realizó un segundo análisis cuantitativo para los elementos de las muestras que se observaran diferencias de concentración graduales con la distancia entre las EM de cada escenario. La determinación cuantitativa de todos los metales seleccionados se realizó mediante espectrofotometría de absorción atómica con cámara de grafito (Perkin Elmer AAnalyst 600), excepto el Hg que se llevó a cabo en un analizador elemental (DMA Milestone).

- **Determinación de antibióticos y pesticidas**

Se realizó un sondeo de la presencia de antibióticos y pesticidas en las muestras de laminaria transplantada en dos EE de las dos LBFF, las situadas a 50 m y 800 m del foco, esperando encontrar las concentraciones máximas y mínimas del gradiente.

Se analizaron los antibióticos mas utilizados en el cultivo de peces planos: amoxicilina, oxitetraciclina, acido oxilínico, flumequina, sulfadiazina, ampicilina y streptomicina. Respecto a los pesticidas, al tener desconocimiento de cuales podían estar presentes, se realizó un screening cualitativo. La determinación de antibióticos y el sondeo de pesticidas se realizaron mediante espectrometría de masas en tándem (MS/MS) acoplada a un HPLC. Previamente se prepararon disoluciones de NaOH 10 mM y acetonitrilo/agua (50:50) para las fases móviles, agua con 0.2% de ácido fórmico y acetonitrilo con 0.1% de ácido fórmico. Para las disoluciones patrón se utilizó el material de referencia y todas las muestras se diluyen en agua de mar, se filtran (0,2µm) y se inyectan en el HPLC-MSMS dotado de una columna Zorbax Eclipse Plus, 2.1 x 30 mm C18, 1.8 µ.

Resultados y discusión

Análisis cualitativo de metales y metaloides

Como una primera aproximación en la identificación de la presencia de posibles contaminantes como resultado de la actividad de las LBFF se hizo un análisis cualitativo de 45 contaminantes traza (Tabla II). En las macroalgas *Fucus vesiculosus* y *Codium tomentosum* nativas no se observan gradientes de contaminación claros para ninguno de los microcontaminantes examinados. En el cultivo, realizado con *S. saccharina* a diferentes distancias del foco vertido, se observan gradientes de bioconcentración de Al, Cu, Hg, Ni y

Pb. En las EE con *Anemone sulcata* nativa se observan reducciones con la distancia en la bioacumulación de Al, Cu, Hg y Ni.

Tabla II.- Resultados de los análisis cualitativo de microcontaminantes expresados como rangos de bioamulación en los diferentes organismos expuestos a los vertidos de las piscifactorías marinas instaladas en tierra (EE) por estaciones de muestreo (EM). Datos expresados en mg l^{-1}.

	Codium tomentosum						
	EE4						
	EM1	EM2	EM3	EM4	EM5	EM6	EM7
Ag	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Al	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
As	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Au	0,01-0,1	<0,01	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01
B	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,1-1	0,01-0,1	0,1-1
Ba	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Be	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Bi	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Ca	>10	>10	>10	>10	>10	>10	>10
Cd	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Co	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Cr	<0,01	<0,01	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01
Cs	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Cu	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,1-1	0,01-0,1	0,01-0,1
Fe	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Ga	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	0,01-0,1
Ge	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Hg	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
In	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
K	>10	1-10	1-10	>10	>10	1-10	>10
Li	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Mg	>10	>10	>10	>10	>10	>10	>10
Mn	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Mo	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
∂^{15}N	5,1	9,93	9,84	9,3	8,14	5,92	5,57
Na	>10	>10	>10	>10	>10	>10	>10
Ni	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,1-1	0,01-0,1	0,01-0,1
P	>10	>10	>10	>10	>10	>10	>10
Pb	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Pd	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Pt	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Rb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sc	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Se	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Si	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Sn	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sr	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Te	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Ti	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,1-1
V	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
W	<0,01	0,1-1	<0,01	0,1-1	0,1-1	<0,01	0,01-0,1
Y	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Zn	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Zr	<0,01	<0,01	<0,01	<0,01	0,1-1	<0,01	<0,01

Tabla II.- (Continuación)

	Saccharina sacharina							
	EE4			EE1				
	EM1	EM3	EM5	EM1	EM2	EM3	EM4	EM5
Ag	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Al	1-10	0,1-1	0,1-1	1-10	1-10	0,1-1	0,1-1	0,1-1
As	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Au	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
B	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Ba	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1
Be	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Bi	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Ca	>10	>10	>10	>10	>10	>10	>10	>10
Cd	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Co	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Cr	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Cs	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1
Cu	1-10	1-10	0,1-1	0,1-1	1-10	1-10	0,1-1	0,1-1
Fe	1-10	1-10	0,1-1	1-10	1-10	1-10	1-10	0,1-1
Ga	0,01-0,1	0,01-0,1	0,01-0,1	0,1-1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Ge	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Hg	0,01-0,1	0,01-0,1	0,1-1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
In	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1
K	>10	>10	>10	>10	>10	>10	>10	>10
Li	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Mg	>10	>10	>10	>10	>10	>10	>10	>10
Mn	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Mo	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	0,01-0,1	<0,01	<0,01	<0,01
$\partial^{15}N$	7,3	7,2	6,2	4,3	7,1	7,3	8,2	1,9
Na	>10	>10	>10	>10	>10	>10	>10	>10
Ni	0,1-1	0,1-1	0,01-0,1	0,01-0,1	0,1-1	0,1-1	0,01-0,1	0,01-0,1
P	>10	>10	>10	>10	>10	>10	>10	>10
Pb	0,01-0,1	0,1-1	0,01-0,1	0,1-1	0,1-1	0,01-0,1	0,01-0,1	0,01-0,1
Pd	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Pt	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Rb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sb	<0,01	<0,01	<0,01	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1
Sc	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	<0,01	<0,01
Se	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Si	1-10	1-10	0,1-1	1-10	1-10	1-10	1-10	0,1-1
Sn	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sr	1-10	>10	>10	1-10	1-10	1-10	1-10	1-10
Te	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Ti	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
V	<0,01	<0,01	<0,01	0,01-0,1	<0,01	<0,01	<0,01	<0,01
W	0,01-0,1	<0,01	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1
Y	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Zn	1-10	1-10	1-10	1-10	1-10	1-10	1-10	0,1-1
Zr	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01

Tabla II.- (Continuación)

	Fucus vesiculosus			
	EE2			
	EM1	EM2	EM3	EM4
Ag	<0,01	<0,01	<0,01	<0,01
Al	0,1-1	1-10	0,1-1	0,1-1
As	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Au	<0,01	0,01-0,1	<0,01	<0,01
B	0,1-1	0,1-1	0,1-1	0,1-1
Ba	0,1-1	0,1-1	0,1-1	0,1-1
Be	<0,01	<0,01	<0,01	<0,01
Bi	0,1-1	0,1-1	0,1-1	0,1-1
Ca	>10	>10	>10	>10
Cd	<0,01	<0,01	<0,01	<0,01
Co	<0,01	0,01-0,1	<0,01	<0,01
Cr	<0,01	0,01-0,1	<0,01	<0,01
Cs	0,1-1	0,1-1	0,1-1	0,1-1
Cu	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Fe	0,1-1	1-10	1-10	0,1-1
Ga	0,01-0,1	0,1-1	0,01-0,1	0,01-0,1
Ge	<0,01	<0,01	<0,01	<0,01
Hg	<0,01	<0,01	<0,01	<0,01
In	0,1-1	0,1-1	0,1-1	0,1-1
K	>10	>10	>10	>10
Li	<0,01	0,01-0,1	<0,01	<0,01
Mg	>10	>10	>10	>10
Mn	0,1-1	1-10	1-10	0,1-1
Mo	0,1-1	0,1-1	0,1-1	0,1-1
$\partial^{15}N$	6,37	8,44	7,89	6,22
Na	>10	>10	>10	>10
Ni	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
P	>10	>10	>10	>10
Pb	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Pd	<0,01	<0,01	<0,01	<0,01
Pt	<0,01	<0,01	<0,01	<0,01
Rb	<0,01	<0,01	<0,01	<0,01
Sb	<0,01	<0,01	0,01-0,1	0,01-0,1
Sc	<0,01	<0,01	<0,01	<0,01
Se	1-10	1-10	1-10	1-10
Si	0,1-1	1-10	0,1-1	0,1-1
Sn	<0,01	<0,01	<0,01	<0,01
Sr	>10	>10	>10	>10
Te	<0,01	<0,01	<0,01	<0,01
Ti	0,1-1	>10	0,1-1	0,1-1
V	<0,01	<0,01	<0,01	<0,01
W	0,1-1	0,1-1	0,01-0,1	0,01-0,1
Y	<0,01	<0,01	<0,01	<0,01
Zn	0,1-1	1-10	0,1-1	0,1-1
Zr	<0,01	0,1-1	<0,01	<0,01

Tabla II.- (Continuación)

	Anemonia sulcata							
	EE4					EE2		
	EM1	EM2	EM3	EM4	EM5	EM1	EM2	EM4
Ag	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Al	1-10	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
As	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Au	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
B								
Ba	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Be	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Bi	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Ca	>10	>10	>10	>10	>10	>10	>10	>10
Cd	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Co	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	<0,01
Cr	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Cs	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Cu	1-10	1-10	1-10	1-10	1-10	1-10	0,1-1	0,1-1
Fe	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Ga	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1	0,01-0,1
Ge	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Hg	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01
In	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
K	>10	>10	>10	>10	>10	>10	>10	>10
Li	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Mg	>10	>10	>10	>10	>10	>10	>10	>10
Mn	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Mo	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
$\partial^{15}N$	10,38	9,6	9,92	9	8,08	8,81	7,7	8,09
Na	>10	>10	>10	>10	>10	>10	>10	>10
Ni	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1
P	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Pb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Pd	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Pt	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Rb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sb	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sc	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Se	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Si	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Sn	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Sr	0,1-1	0,1-1	1-10	0,1-1	1-10	0,1-1	0,1-1	0,1-1
Te	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Ti	1-10	0,1-1	1-10	1-10	1-10	0,1-1	0,1-1	0,1-1
V	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
W	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,01-0,1	0,01-0,1
Y	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Zn	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Zr	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1

Tabla II.- (Continuación)

	Mytilus galloprovincialis													
	EE2			EE3			EE4		EE5		EE7		EE8	
	EM1	EM2	EM3	EM1	EM2	EM3	EM1	EM2	EM1	EM2	EM1	EM2	EM1	EM2
Ag	<0,01	<0,01	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Al	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
As	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Au	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
B	1-10	1-10	0,1-1	<0,01	<0,01	<0,01	<0,01	<0,01	0,1-1	0,1-1	0,1-1	<0,01	<0,01	<0,01
Ba	0,01-0,1	0,01-0,1	<0,01	<0,01	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Be	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Bi														
Ca	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Cd	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Co	<0,01	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1
Cr	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Cs														
Cu	0,1-1	0,1-1	0,1-1	0,01-0,1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Fe	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Ga	0,01-0,1	<0,01	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1	<0,01	<0,01
Ge	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Hg	0,1-1	0,01-0,1	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
In	0,01-0,1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,01-0,1	0,1-1	0,1-1	0,01-0,1	0,1-1
K	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Li	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Mg	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Mn	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Mo	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
$\partial^{15}N$	8,1	8,4	7,6	8,9	6,8	7,1	9,6	9	10,2	10,3	8,1	7,7	9,3	8,8
Na	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Ni	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
P														
Pb	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
Pd	<0,01	<0,01	<0,01	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Pt														
Rb														
Sb	<0,01	0,1-1	0,1-1	<0,01	<0,01	<0,01	<0,01	<0,01	0,1-1	0,1-1	<0,01	<0,01	0,1-1	0,1-1
Sc	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Se	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Si	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Sn	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Sr	0,1-1	1-10	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Te														
Ti	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,01-0,1
V	<0,01	<0,01	<0,01	<0,01	<0,01	0,01-0,1		<0,01	0,01-0,1	0,01-0,1	0,01-0,1	0,1-1	<0,01	<0,01
W	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1	0,1-1
Y	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Zn	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10	1-10
Zr	0,01-0,1	0,01-0,1	0,01-0,1	<0,01	<0,01	<0,01	<0,01	<0,01	0,01-0,1	<0,01	<0,01	0,01-0,1	0,01-0,1	<0,01

Análisis antibióticos y pesticidas

En la Tabla III se recogen los resultados obtenidos en las muestras de *S. saccharina* para los antiobióticos analizados. La cuantificación de la sulfadiazina está por debajo del límite de cuantificación en las dos LBFF. En la EM más cercana al vertido se observan bioacumulaciones de ácido oxilínico, amoxicilina y oxitretraciclina e inferiores de flumequina. Se observan valores superiores de ácido oxilínico y flumequina en la granja EE1 y de amoxicilina y oxitetraciclina en EE4. Las diferencias entre EM se hace más evidente para amoxicilina alcanzando un orden de magnitud. También se observan diferencias de bioconcentración entre granjas.

Tabla III.- Bioconcentración de antibióticos (ácido oxilínico, amoxicilina, flumequina, oxitetraciclina, sulfadiazina), en muestras de *S. Saccharina* situadas a 50 m (EM1) y 800 m (EM5) del efluente de dos granjas de rodaballo instaladas en tierra (LBFF).

		Antibióticos (ng/g)				
LBFF	EM	Acido Oxilínico	Amoxicilina	Flumequina	Oxitetraciclina	Sulfadiazina
EE4	EM1	0,06	163,00	0,29	7,93	<0
	EM5	<0	<0	2,50	2,52	<0
EE1	EM1	0,52	46,70	0,50	6,07	<0
	EM5	<0	<0	1,06	2,50	<0

En el screening de pesticidas en la granja EE4 se detectó la presencia de: propacloro, prometrina, prometon, difenilamina, clorotalonil y malation. En la EE1 se detectaron: tebutam, propacloro, prometrina, prometon, fention, clorotalonil. En ambas granjas se observó la aparición de: propacloro, prometrina, prometon, difenilamina y clorotalonil.

Análisis cuantitativo

Tras los resultados del análisis químico cualitativo se cuantificó en todos los biomonitores la bioacumulación de Hg. En la figura 3 se puede observar como existe un gradiente claro de disminución de Hg con la distancia. Este gradiente es más evidente en los transplantes de laminaria y en las anémonas nativas.

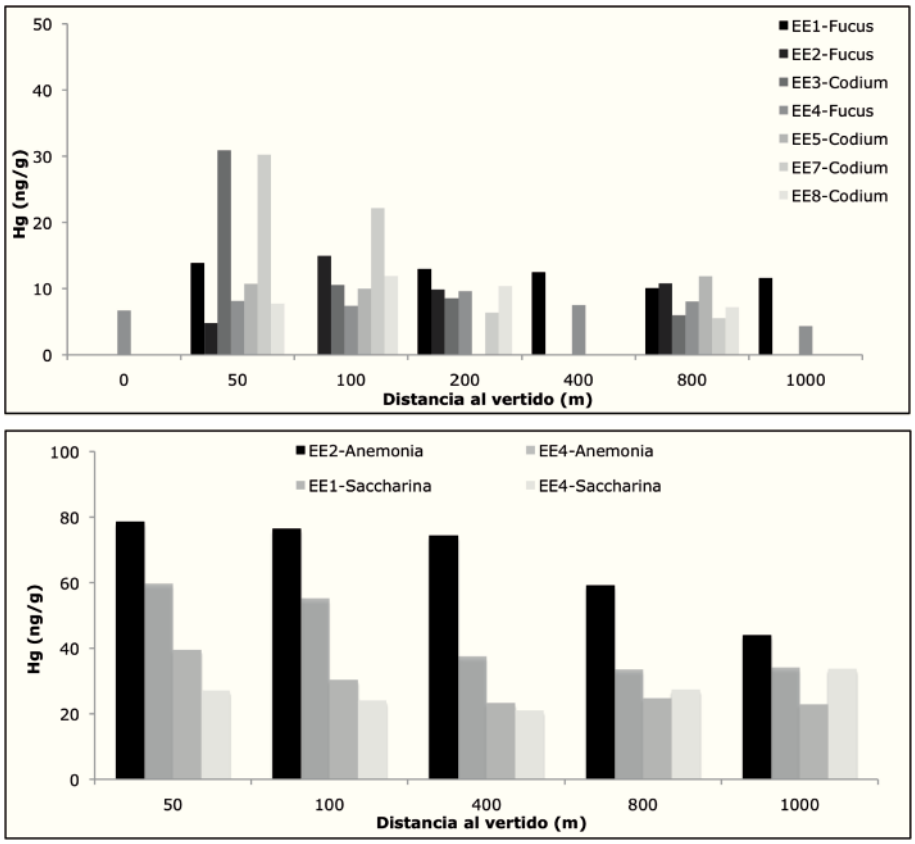


Figura 3.- Mercurio (Hg) bioconcentrado en las macroalgas (*Fucus sp., C. tomentosum., S. sacharina* y *A. sulcata*) en relación con la distancia al punto de vertido de las piscifactorías marinas instaladas en tierra (EE) utilizadas en este estudio.

Análisis de $\delta^{15}N$ y relación con los microcontaminantes bioacumulados

Mientras que en las macroalgas *F. vesiculosus* y *C. tomentosum* nativas no se observaron gradientes de contaminación metálicos estos si son evidentes para la señal isotópica $8\delta^{15}N$). Se observa un incremento de $\delta^{15}N$ en anémonas y macroalgas, sobretodo en las recolectadas a unos 100 m del emisario. Este enriquecimiento disminuye progresivamente con la distancia al foco alcanzando los niveles control en la mayoría de los escenarios a partir de los 500 m del emisario. En este sentido, el gradiente de la $\delta^{15}N$ observado en EE4 con *C. tomentosum* es ejemplar.

Se ha encontrado correlación significativa entre el contenido corporal de Ni en *A. sulcata* y la señal $\delta^{15}N$ en las dos LBFF estudiadas (Fig. 4). Estos resultados confirman que la $\delta^{15}N$ en macroalgas es un buen descriptor de la extensión y grado de influencia de los efluentes de las LBFF, resultado de la carga contaminante y de la capacidad dispersiva del

medio. Además, la señal $\delta^{15}N$ informa sobre la fracción del N biodisponible, una guía para la evaluación del riesgo de eutrofización (Rey-Asensio *et al.,* 2009).

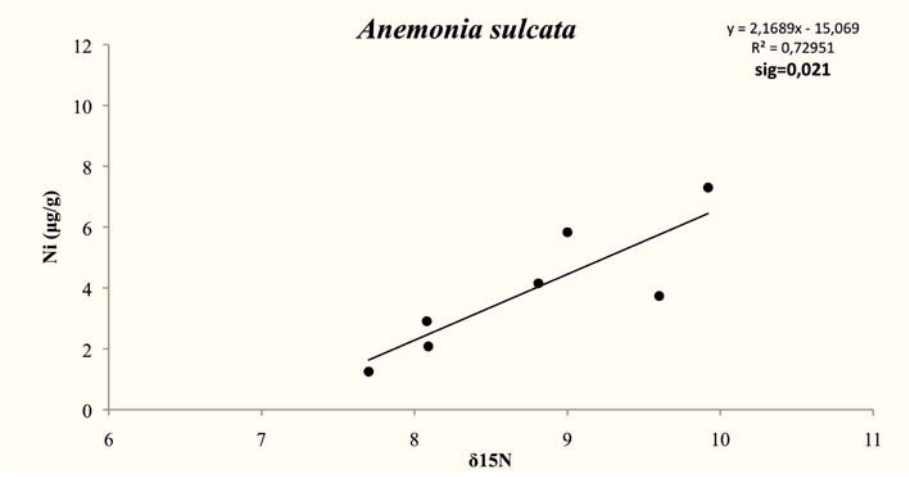


Figura 4.- Relación entre la señal isotópica ($\delta^{15}N$) y la bioacumulación de Ni en *Anemonia sulcata* recogida en el área de influencia de dos granjas marinas instaladas en tierra.

Conclusiones

La observación de gradientes de contaminación metálicos no implica "a priori" toxicidad, ni riesgo ecológico, solo son marcadores o *descriptores de exposición*.

No se ha encontrado ningún microcontaminante común a todos los escenarios analizados que pueda utilizarse como *descriptor tipo* claro en los protocolos de vigilancia de las granjas de acuicultura instaladas en tierra.

Las bioconcentraciones de antibióticos encontradas *S. Saccharina* se encuentran por debajo de los límites establecidos para la alimentación humana.

Se confirma la señal isotópica $\delta^{15}N$ con un buen descriptor un buen descriptor de la extensión y grado de influencia de los efluentes de las LBFF.

Agradecimientos

Este estudio fue financiado por el Plan Nacional de Cultivos Marinos (JACUMAR, 2008) dentro del proyecto interautonómico: "Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos y medidas para estudios ambientales en acuicultura (INDAQUA)".

Bibliografía

Carballeira C., Martin-Díaz ML., delValls A., Carballeira A. (enviada): Designing an integrated environmental monitoring plan for marine land-based fish farm. *Aquaculture Environmental Interactions.*

Cloern J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. *Marine Ecology Progress Series,* 210: 223-253.

Ervik A., Hansen P.K., Aure J., Stigebrandt A., Johannessen P., Jahnsen T. 1997. Regulating the local environmental impact of intensive marine fish farming I. The concept of the MOM system (Modelling- Ongrowing fish farms- Monitoring). *Aquaculture,* 158: 85-94.

Fernandes T.F., Eleftheriou A., Ackefors H., Eleftheriou M., Ervik A., Sanchez-Mata A., Scanlon T., White P., Cochrane S., Pearson T.H. nd Read P.A. 2002. The management of the environmental impacts of aquaculture. Scottish Executive, Aberdeen, UK. 88p.

Phillips D.J.H. 1977. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments: A review. *Environmental Pollution* 13: 281-317.

Read P. and Fernandes T. 2003. Management of environmental impacts of marine aquaculture in Europe. *Aquaculture,* 226:139-163.

Rey-Asensio A., Viana I.G., Carballeira C. y Carballeira A. 2009. $\delta^{15}N$ en macroalgas como marcador del área de influencia de los vertidos de piscifactorías marinas instaladas en tierra. En: *XVII Simpósio de Botanica Criptogámica*. (23-26 de septiembre, 2009 Tomar, Portugal).

Viana I. G., Fernández J.A., Aboal J.R., Carballeira A. (2011): Measurement of $\delta^{15}N$ in macroalgae stored in an environmental specimen bank for regional scale monitoring of eutrophication in coastal areas. *Ecological Indicators*, 11: 888-895

J Appl Phycol
DOI 10.1007/s10811-012-9843-z

$\delta^{15}N$ values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms

Carlos Carballeira · Inés G. Viana · Alejo Carballeira

Received: 31 January 2012 / Revised and accepted: 8 April 2012

Abstract The nitrogen isotope ratio ($\delta^{15}N$) in tissues of native macroalgae was evaluated as a means of indicating the intensity and spatial extent of organic contamination due to disposal of waste from land-based marine fish farms (LBMFFs). Three species of macroalgae from the genus *Fucus* and the green macroalgae *Codium tomentosum* were selected for study. The study was carried out at seven flat marine fish farms located in Galicia (NW Spain). Tests were carried out to determine the intra-annual variation in $\delta^{15}N$ values and any differences between selected macroalgae. The $\delta^{15}N$ values enrichment was observed close to the disposal point, and $\delta^{15}N$ values varied more widely throughout the year (±5.57 ‰) at sites affected by the marine fish farm effluent compared to natural conditions (±2 ‰). No significant differences in the isotopic signals were observed in the different species studied (standard major axis). The $\delta^{15}N$ values of macroalgae may be an ideal means of detecting the presence of LBMFFs effluents.

C. Carballeira (✉)
Marine Ecotoxicology, Área de Química Física,
Facultad de Ciencias del Mar, Universidad de Cádiz,
Cadiz 11510, Spain
e-mail: carlos.carballeira@uca.es

I. G. Viana
Instituto Español de Oceanografía,
Centro Costero de A Coruña, 130,
A Coruña 15080, Spain
e-mail: ines.gonzalez@co.ieo.es

A. Carballeira
Ecotoxicología, Área de Ecología, Facultad de Biología,
Universidad de Santiago de Compostela,
Santiago de Compostela 15782, Spain
e-mail: alejo.carballeira@usc.es

Keywords *Fucus* · *Codium tomentosum* · Pollution · Monitoring · Bioconcentration · Aquaculture · Eutrophication

Introduction

Aquaculture activity has increased greatly in coastal areas throughout the world in recent years. This has been possible due to the introduction of new technologies, the increased availability of suitable sites, improvements in food technology, improved understanding of the biology of the species farmed, increased water quality within farming systems and the increased demand for fish products (Read and Fernandes 2003). Worldwide, the sector has grown since 1970 and in Europe represents almost 4 % of aquaculture production worldwide (FAO 2010). In Galicia (NW Spain), the region under consideration in the present study, the sector represents 86.95 % of the Spanish aquaculture production (APROMAR 2011). The Galician Aquaculture Plan contemplates, with extensions and new installations, occupying some 3.10^6 m^2 in land-based marine fish farms (LBMFFs), with an envisaged intensive annual production of flatfish of 30,000 t $year^{-1}$.

This intensive development has been accompanied by an increase in the environmental impact of these activities (Ervik et al. 1997; Fernandes et al. 2001). Eutrophication is one of the most important of these impacts because of the high levels of ammonium and nitrates that are released as a result of aquaculture activity to coastal communities, which are often limited by nitrogen (Cloern 2001). A variety of indices have been developed to quantify the extent of eutrophication brought about by nitrogen loading, as taxonomic shifts and changes in abundance of producers and consumers take place (McClelland et al. 1997). However, the disadvantage of these indices is that the effects are only

Published online: 10 May 2012

detected when environmental disturbance has already occurred. On the other hand, the water quality parameters traditionally analyzed, i.e. dissolved nutrients, appear to fluctuate significantly over short periods of time (Wolanski et al. 2000). This implies that quantification is difficult and is associated with a very costly intensity of sampling (Dalsgaard and Krause-Jensen 2006; Sarà et al. 2004).

Analysis of the ratio of stable isotopes of nitrogen ($\delta^{15}N$) appears to be an ideal alternative, as variations in the relative abundance of the isotopes can be detected before coastal communities undergo alterations in structure and function. The method is based on the fact that nitrogen has two stable isotopes, a light isotope, ^{14}N, and a heavier isotope, ^{15}N, which occur at a constant proportion in the atmosphere, respectively, 99.635 % and 0.365 % (Nier 1950). Isotopic abundance is reported on a delta scale (δ), which indicates the deviation (expressed in per mille) of the isotopic composition of a sample from an internationally accepted standard, the air (e.g. Robinson 2001).

However, this proportion varies according to the different metabolic routes that a molecule follows, as the diverse reactions produce different fractioning of the heavy isotope (^{15}N) (Struck 2012). Significant variations in the natural abundance of ^{15}N in marine organisms were first documented in the 1950s (Hoering 1955), but the earliest specific studies of nitrogen isotopic abundance in marine systems were carried out in the mid 1960s (Minagawa and Wada 1984; Miyake and Wada 1967; Wada et al. 1975; Wada and Hattori 1976). Tracking these variations in the environment was found to have many novel applications in ecological studies, e.g. migration studies, trophic chains and paleoecological studies (Michener and Lajtha 2007; Struck 2012). Such applications included analysis of $\delta^{15}N$ to indicate sources of nitrogen in marine systems, although this was not applied until the 1990s (Costanzo et al. 2001; Dailer et al. 2012; Lamb et al. 2012; Mattern et al. 2011; Rogers 1999; Savage 2005; Umezawa et al. 2002). The use of stable isotope analysis to trace organic contamination is based on the fact that different anthropogenic sources of nitrogen alter the baseline levels of $\delta^{15}N$ values of marine systems (Heaton 1986), so that the method can be used to trace and quantify the nitrogen inputs (Filgueira and Castro 2011; Struck 2012).

In marine systems, different matrices (biotic and abiotic samples) can be used for isotope analysis (Lamb et al. 2012). However, the advantage of using biotic samples rather than environmental samples (i.e. water and sediment) is that information about the bioavailable fraction, i.e. that associated with the potential risk of eutrophication, can be obtained (Lapointe et al. 2007; Yang et al. 2008). Amongst the biotic samples used, macroalgae have all the main characteristics required for biomonitoring metals (Carballeira et al. 2000; Viana et al. 2010) and nutrients (Villares and Carballeira 2003, 2004, 2006). Macroalgae are very sensitive to changes in water quality induced by human activities and have been used as biomonitors of $\delta^{15}N$ values of many environmental studies (Dailer et al. 2012; Deutsch and Voss 2006; Lamb et al. 2012; Lapointe 2010; Riera et al. 2000; Savage 2005; Savage and Elmgren 2004; Viana et al. 2011). Moreover, macroalgae absorb dissolved inorganic nitrogen in the water (Lobban and Harrison 1994) and accumulate it in their tissues without mobilising it, and are therefore not affected by sporadic episodes. In this way, the $\delta^{15}N$ values of macroalgae accurately reflect the mean concentration of nitrogen in water sources (McClelland et al. 1997; Vosz and Struck 1997), and the long turnover time allows temporal integration of the ^{15}N source signal (Costanzo et al. 2001).

For all of these reasons, using the $\delta^{15}N$ values of macroalgae and other autotrophic organisms to detect the presence of effluents from land-based aquaculture (Costanzo et al. 2001; Jones et al. 2001; Lin and Fong 2008; Vizzini and Mazzola 2004), and of aquaculture settlements in general (Dolenec et al. 2006; García-Sanz et al. 2010; Lojen et al. 2005), has become more popular in the last few years. Fish farm waste generally has significantly higher $\delta^{15}N$ values because the heavier isotope ^{15}N remains in the effluent source while the lighter isotope ^{14}N is volatilized through microbial processes (Heaton 1986; Macko and Ostrom 1994; Van Dover et al. 1992). However, further basic information is required in order to establish standardized protocols.

There is a lack of information about aspects such as interspecific differences or the natural seasonal variation of $\delta^{15}N$ values of macroalgae (Lapointe et al. 2007). Furthermore, nitrogen naturally occurs in the marine environment, so it is important to evaluate the differences between the natural (background) $\delta^{15}N$ values of macroalgae and those affected by waste disposal. Different species of native or transplanted macroalgae have been used in marine fish farm studies, but always on offshore fish farms (cages) or shrimp ponds, with very scarce reference to any studies of the effects of LBMFFs (Vizzini and Mazzola 2004).

The aim of the present study was to test the effectiveness of the $\delta^{15}N$ values of macroalgae for assessing the detection of LBMFFs effluents and the magnitude of nutrient enrichment. In addition, in order to design better protocols for biomonitoring these industries, the following aspects related to $\delta^{15}N$ values of macroalgae were considered: (a) interspecific differences of $\delta^{15}N$ values between species, (b) annual variability in marine fish farm loads, and (c) comparison between the $\delta^{15}N$ of background and those observed near farms.

Materials and methods

Macroalgal surveys were conducted in Galicia (NW Spain), in July 2008. Seven LBMFFs, situated in coastal areas with different hydrodynamic conditions and in absence of other nearby sources of organic contamination, were selected

(Fig. 1). All of these were dedicated to the cultivation of flatfish, basically turbot (*Psetta maxima*). At each of the seven marine fish farms, between three and eight locations were located on an exponential gradient (approximately 0 to 1,500 m) from the emission point and in the direction of the predominating current (Fig. 1). The mean production on the farms fluctuated between 44 and 2,250 t year^{-1} (Table 1). The mean concentration (n=22) of total nitrogen in the input and output water from the LBMFFs under study was 1.34±0.17 and 1.78±0.24 mg L^{-1}, respectively, whereas the concentration of ammoniacal nitrogen was 2.33±1.35 and 2.42±1.56 mg L^{-1}, respectively.

The surveys were carried out at low tide in the mesolittoral zone. Each location included 20 m of coastline. At each location, more than 30 specimens of macroalgae attached to the substrate were collected systematically, following a zigzag line, with the aim of covering the degree of variability in the inter-individual concentrations. The specimens were combined to make a composite sample for each location, washed in situ with seawater and transported at 4°C to the laboratory where they were stored at −30°C (for less than 1 month).

The material was defrosted at room temperature before processing. It was then washed carefully with abundant filtered seawater in successive stages, to remove, as efficiently as possible, any sediment and epiphytes; old and damaged parts of the plants were discarded. The distal 3 cm of the shoots were used to determine the concentrations; these portions were separated from the rest of the plant with a glass spatula, and the samples were homogenized in a laboratory blender. All of the material was dried at 45°C in a forced air oven and homogenized in an

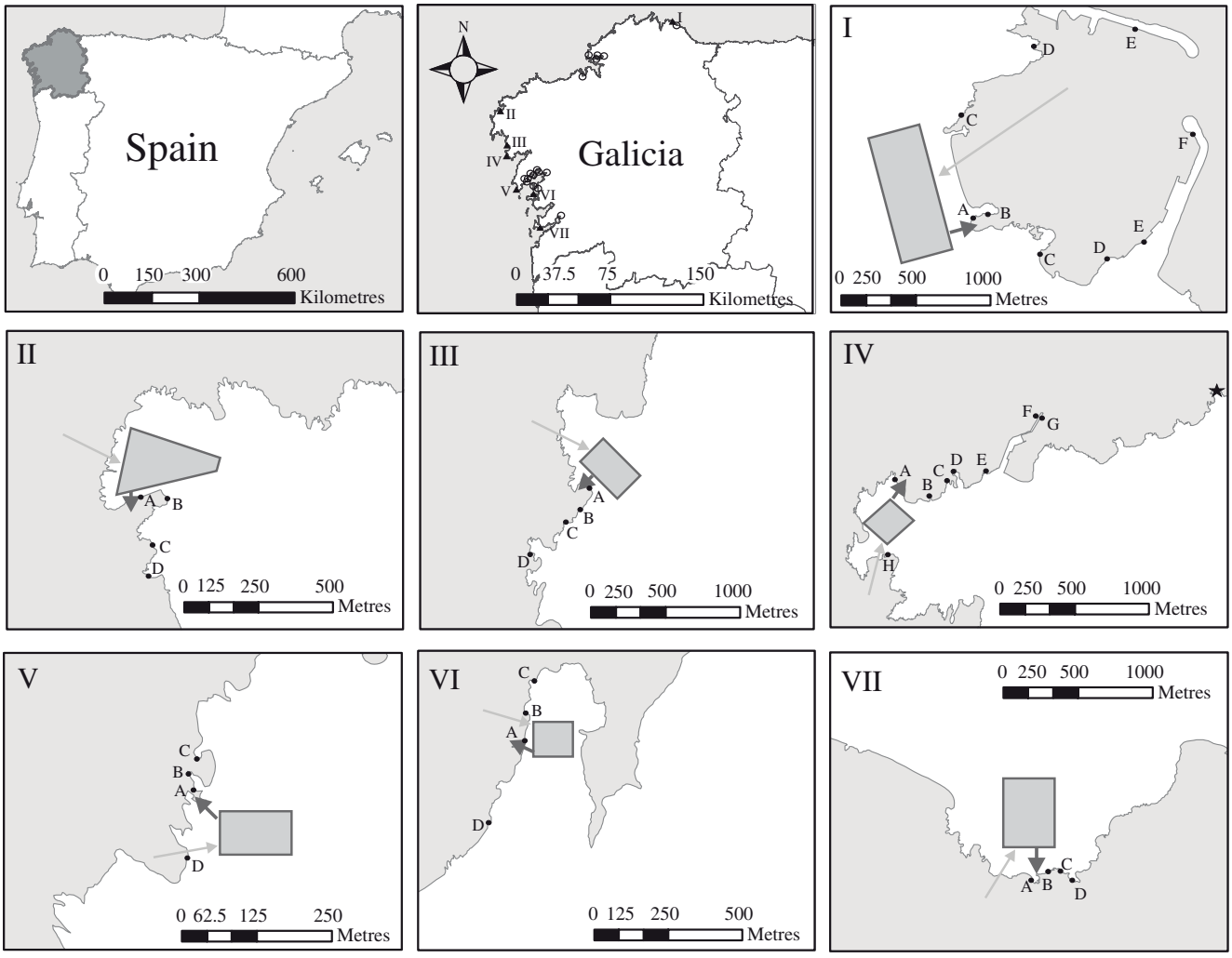


Fig. 1 Map showing the location of Galicia in NW Spain, the location of the study areas in Galicia (*solid triangles*) and the location where more than one species coexist are represented by *opene circles*. *Enlargements* show the surroundings of the land-based marine fish farms studied (different scales). In the enlarged figures of the marine fish farms, the sampling locations are shown in *letters* along a down current gradient from the point of discharge to 1,500 m (Table 1). The *solid star* (fish farm IV) indicates the clean site sampled monthly. The *arrows* indicate the input (*grey*) and output (*black*) of water from the land-based marine fish farms, which are shown as *grey areas*

Table 1 $\delta^{15}N$ values of (A) *Fucus vesiculosus* and (B) *Codium tomentosum* from the 7 seven land -based marine fish farms at the respective sampling locations (A–-H) arranged in increasing distance from the point of effluent discharge and annual production at the time of sampling. The bold and underlined values in (A) represent locations where *F. spiralis* and *F. serratus* were sampled, respectively. The shaded areas represent locations < 50 m from the point of effluent discharge

The *bold and underlined* values in (A) represent locations where *F. spiralis* and *F. serratus* were sampled, respectively. The values in *italics* represent locations <50 m from the point of effluent discharge

Farm	Production (t year^{-1})	A	B	C	D	E	F	G	H
(A) *Fucus vesiculosus*									
Fish farm I west	2,250	*5.24*	*5.62*	11.17	9.77	9.57	––	––	––
Fish farm I east				**9.25**	––	10.41	8.14	––	––
Fish farm II	292	*6.37*	8.44	7.89	6.22	––	––	––	––
Fish farm III	307	*5.11*	8.1	––	5.98	––	––	––	––
Fish farm IV	1,194	––	––	––	––	––	––	5.7	5.72
Fish farm V	347	––	––	––	––	––	––	––	––
Fish farm VI	44	*4.41*	8.29		5.67	––	––	––	––
Fish farm VII	285	*1.27*	8.6	––	––	––	––	––	––
(B) *Codium tomentosum*									
Farm		A	B	C	D	E	F	G	H
Fish farm I west	2,250	––	––	––		8.84	––	––	––
Fish farm I east		––	––	––	10.28	9.11	9.35	––	––
Fish farm II	292	––	––	––	––	––	––	––	––
Fish farm III	307	*2.85*	7.87	6.58	––	––	––	––	––
Fish farm IV	1,194	*5.1*	9.93	9.84	9.3	8.14	5.92	5.57	5.57
Fish farm V	347	*1.7*	*4.9*	5.6	6	––	––	––	––
Fish farm VI	44	*4.77*	8.46	6.41	––	––	––	––	––
Fish farm VII	285	*2.87*	9.24	9.44	8.33	––	––	––	––

ultracentrifugal mill (Retsch ZM 100). Dried samples were stored at room temperature in glass vessels.

Biomonitors

For this study, the macroalgae *Fucus vesiculosus* was selected as the main biomonitor, as it is very abundant in the study area. However, in the relatively small area of influenced by the farms, it is not always possible to find *F. vesiculosus*, so other species that are ubiquitous on the coast of Galicia (*Fucus spiralis* and *Fucus serratus*) were chosen as secondary biomonitors. In those cases in which no members of the Phaeophyceae were found, the chlorophyte *Codium tomentosum* was used, as it is often present in the area of influence of marine fish farms.

Stable nitrogen isotope ($\delta^{15}N$) analysis

Aliquots (3 mg) of the dried samples were weighed and packed into tin capsules (EuroVector). The capsules were stored in a desiccator until $\delta^{15}N$ analysis (carried out in the Unidad de Técnicas Instrumentales de Análisis (UTIA), Servicios de Apoyo a la Investigación, University of A Coruña). The samples were combusted in an elemental analyzer (FlashEA1112: ThermoFinnigan) coupled to an isotopic ratio mass spectrometer (Delta^{plus}: ThermoFinnigan). Acetanilide was used as the reference standard for quantifying the nitrogen content. Calibration of the reference gas for atmospheric ^{15}N was carried out with IAEA-N-1 ($(NH_4)_2SO_4$), IAEA-N-2 ($(NH_4)_2SO_4$) and IAEA-NO-3 (KNO_3) as standards.

The isotopic ratios ($^{15}N/^{14}N$) of the samples were compared with the standard (atmospheric N_2), so that comparable proportions were obtained. The relative abundance of ^{15}N in the sample ($\delta^{15}N$) was calculated from the formula: $\delta^{15}N\ (‰) = [(R_{sample}/R_{standard}) - 1] * 10^3$, where R is the $^{15}N/^{14}N$ ratio. The overall error was determined by use of analytical replicates and constitutes a measure of the precision of the technique, as it is the coefficient between the standard deviation of the replicates and the number of replicates. The overall error of the replicates of 30 samples was 2 %.

Interspecific differences in $\delta^{15}N$ values of macroalgae

The difference between $\delta^{15}N$ values of the four species of macroalgae was evaluated by the following comparisons of pair of species: *F. vesiculosus* and *F. spiralis*, *F. vesiculosus* and *F. serratus*, *F. spiralis* and *F. serratus*, and *F. vesiculosus* and *C. tomentosum*. For this, 12, 8, 6 and 11 locations were used in 2008 and 2009 where each pair of species coexisted (Fig. 1). To study interspecific differences, the model parameters (slopes and elevations) for interspecific bioconcentration regression lines [standard major axis (SMA)] for each pair of species were compared with the line of slope 1 and elevation 0, by use of the *t* statistic. The difference between estimated and hypothesised slope and elevation to the standard error of elevation was thus compared (Warton and Ormerod 2007). The calculations were

carried out with the "smatr" package (Warton and Ormerod 2007), under *R* (R Development Core Team 2008).

Establishment of the regional reference levels of $\delta^{15}N$ values of macroalgae

Data from a prior survey conducted for different purposes were used in the present study. The samples were collected in a regional coastal survey carried out in July 2007 in Galicia, and deposited in the Environmental Specimen Bank of Galicia (ESBG). The sampling locations in the study were located at distances of more than 300 m from drains, ports or industrial facilities, and more than 150 m from the mouths of first- or second-degree rivers. The data from this survey, as well as the methods of sampling and processing the macroalgae deposited in the ESBG, are described by Viana et al. (2011).

Seasonal variability in $\delta^{15}N$ values of macroalgae

The study was conducted at three locations, one of which was located at a supposedly clean site (Fig. 1), and two were located in the surroundings of two different marine fish farms (site C at fish farms II and III, Fig. 1). Samples of *F. vesiculosus* were collected at each of the three locations, and at fish farm III, *F. spiralis* was also sampled. Each location was sampled every month (ten replicates at each location) so that there were a total of 12 mean samples from each location. The experiment began in February 2009 and ended in January 2010.

Results

The individual $\delta^{15}N$ values of macroalgae collected at the different locations are shown in Table 1. The locations are ordered from A to H, being A, the closest, and H the furthest from the waste disposal point. Those locations at which a species other than *F. vesiculosus* was sampled are shown in Table 1. Similar $\delta^{15}N$ values were obtained in different macroalgae sampled at the same S.S [see e.g. fish farm VI (Table 1)]. In all species, there was enrichment of $\delta^{15}N$ values of the macroalgae collected from between 100 and 500 m from the marine fish farms (sites B to D). The $\delta^{15}N$ values decreased gradually with increasing distance from the dumping point, except in some cases in which similar values were maintained at all locations, for both high values (fish farm I) and low values (fish farm V).

The $\delta^{15}N$ values of the different species studied are compared in Fig. 2. There generally were no significant differences between the slopes and elevations of the pairs of data studied and the line of slope 1 ($p>0.05$, null hypothesis accepted); however, the comparison between *F. vesiculosus* and *F. serratus* revealed significant differences between elevations (Fig. 2). The kernel smoothing distribution of the values of $\delta^{15}N$ values of *F. vesiculosus* in the 2007 BEAG survey are shown in Fig. 3; this corresponds to a normal distribution (Shapiro–Wilk and Kolmogorov–Smirnov). The distribution of the combination of all of the macroalgae located nearby the marine fish farms (Table 1) was slightly skewed to the right with respect to the control distribution (Fig. 3).

With regard to the seasonal variation in the $\delta^{15}N$ values of macroalgae, the results showed that $\delta^{15}N$ values did not vary more than 2 ‰ during the year at the control site (shaded areas, Fig. 4). By contrast, the range of variation in $\delta^{15}N$ values of macroalgae affected by waste discharging from the marine fish farms varied by about 5.57 ‰ throughout the year. The highest value of $\delta^{15}N$ was observed in August in both farms and in both species sampled (*F. vesiculosus* and *F. spiralis*). This maximum was not synchronous with that of the control location, which occurred in November (Fig. 4). The variation of $\delta^{15}N$ values throughout the year in the two marine fish farms under study is likely not to enable the detection of farm effluents to be deduced from a single annual sample. Because of this, controls with macroalgae may be carried out during the period July to September (Fig. 4), with the aim of selecting the maximum annual value of $\delta^{15}N$.

Discussion

The concentrations of dissolved elements and the deposition of particulate residues in the surroundings of a marine fish farm largely depend on the relationship between the production rate of the farm and the dispersive capacity of the environment (Carballeira et al. 2012). This relationship varies temporally and spatially and can give rise to multiple environmental interactions that may generate different types of perturbations and responses from the biota (Tello et al. 2010). Bacteria preferentially use ^{14}N; thus, marine fish farm effluents are enriched in ^{15}N (Heaton 1986), which will be uptaken by primary producers. For this reason, the usefulness of $\delta^{15}N$ measured in macroalgae as monitoring biotool of intensive LBMFFs was assessed (Table 1).

To date, the analysis of $\delta^{15}N$ values has been used to evaluate the effects of disposal of aquaculture activities with red macroalgae and one brown macroalgal species (Jones et al. 2001; Lin and Fong 2008; Vizzini and Mazzola 2004). However, the results obtained in the present work confirmed that macroalgae of the genera *Fucus* and *C. tomentosum* successfully detected the extent of the waste. The isotopic signal was enriched in the macroalgae close to the disposal point, and the maximum values were even higher than in previous studies (Table 2). The highest macroalgal $\delta^{15}N$ values of samples affected by the effluents from the turbot farms ranged from

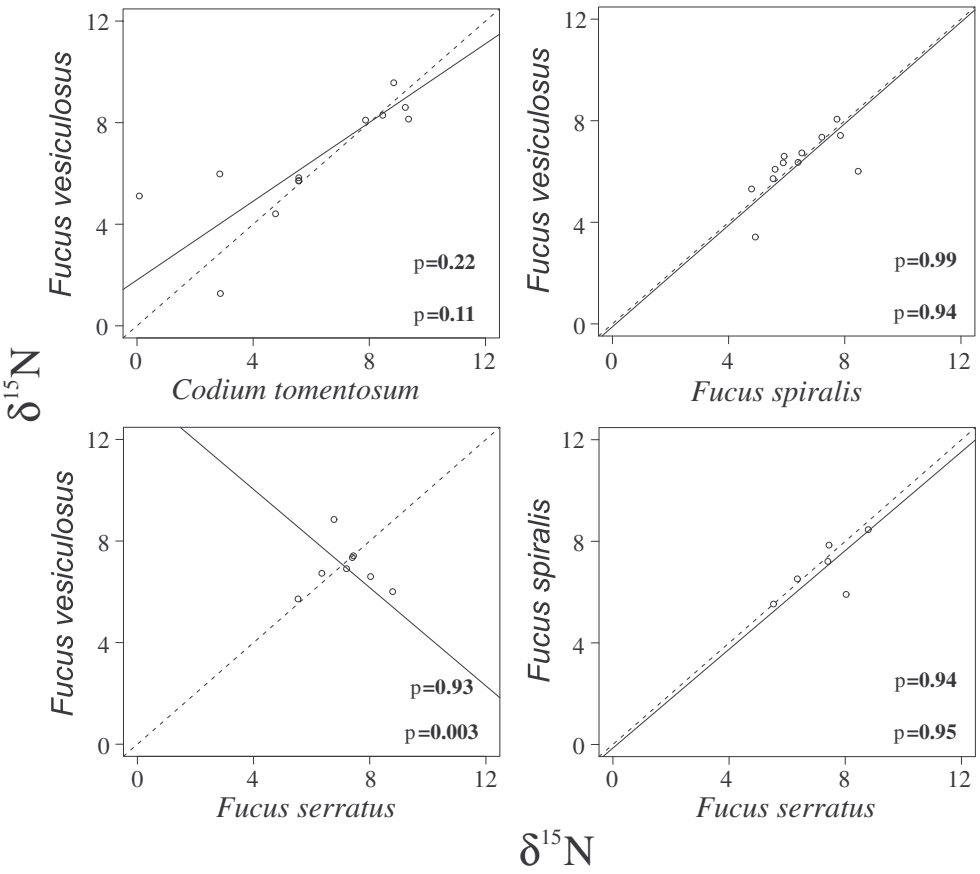


Fig. 2 Standard major axis lines of the $\delta^{15}N$ values (‰) (per mille) from the pairs of species studied are represented by *fine lines*,; *dashed lines* represent a line with a slope equal to 1. The value of the significance of the comparison of the slopes and elevations are shown (in *bold*) in each case

7.8 ‰ to 11.17 ‰ (Table 1), whereas values reported in the literature vary between 4.2 ‰ and 7.1 ‰ in red macroalgae from the surroundings of shrimp farms (Costanzo et al. 2004; Jones et al. 2001; Lin and Fong 2008). In LBMFFs, Vizzini and Mazzola (2004) observed maximum macroalgal $\delta^{15}N$ values of 6.3±1.2 ‰. Isotopic enrichment in macroalgae in the surroundings of LBMFFs may be related to isotopic differences in the fish food used. The mean value of $\delta^{15}N$ values of the pelleted food provided to the turbot (Nutreco Aquaculture, Skretting) was 7.43±0.45 ‰, while the value of the pelleted food used on shrimp farms was 6.3±0.6 ‰ (Landrum and

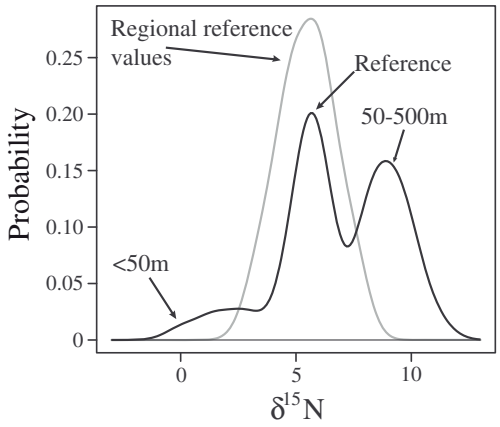


Fig. 3 Distribution of the regional reference values of $\delta^{15}N$ in *Fucus vesiculosus* (BEAG, 2007 survey) and distribution of the macroalgae (genus *Fucus* and *Codium tomentosum*) collected around the 7 seven land -based marine fish farms, estimated by kernel smoothing

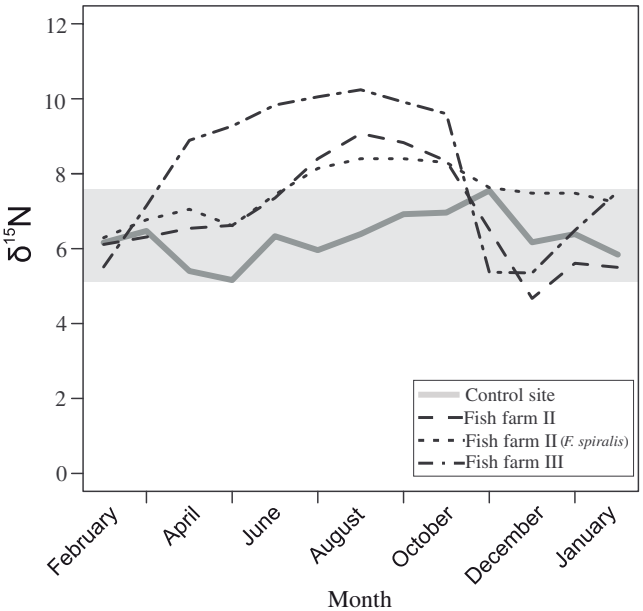


Fig. 4 Monthly variations in $\delta^{15}N$ (‰) (per mill) in *Fucus vesiculosus* (site C at fish farm II and fish farm III and at a control site, *solid star* at fish farm IV, Fig. 1) and *Fucus spiralis* (site C at fish farm II, Fig. 1) for the period February 2009 to January 2010

Table 2 Reported $\delta^{15}N$ values of marine macroalgae influenced by fish and shrimp farm effluents

Species cultivated	Production (t year^{-1})	Macroalgae species	Type of test (duration)	$\delta^{15}N$ control (‰)	$\delta^{15}N$ source (‰) (distance, m)	Location	Reference
Penaeus merguiensis[a]	––	*Catenella nipae*	Transplant (4 days)	3.3±0.7	4.8±0.6 (0––2000)	North Queensland, Australia	Burford et al. (2003)
Penaeus merguiensis[a]	––	*Catenella nipae*	Transplant (4 days)	≈3	4.2––4.3 (0)	Australia	Costanzo et al. (2004)
Sparus aurata, *Dicentrarchus labrax*[b]	375	*Asparagopsis taxiformis*	Transplant (4 days)	3.9±0.2	4––5 (100)	Canary Islands, Spain	García-Sanz et al. (2010)in press
Sparus aurata, *Dicentrarchus labrax*[b]	375	*Stypopodium zonale*	Transplant (4 days)	2.5±0.2	3––4 (0)	Canary Islands, Spain	García-Sanz et al. (2010)in press
Sparus aurata[b]	800	*Cystoseira mediterranea*	Transplant (4 days)	6.1±0.3	6––7 (0)	Catalonia, Spain	García-Sanz et al. (2010)in press
Thunnus thynnus[b]	350	*Dictyopteris polypodioides*	Transplant (4 days)	1.7±0.1	≈4 (0)	Murcia, Spain	García-Sanz et al. (2010)in press
Penaeus japonicus[a]	––	*Catenella nipae*	Native	2.9	8.6 (mid creek)	Moreton Bay, Australia	Jones et al. (2001)
Penaeus stylirostris[a]	12	*Acanthophora spicifera*	Transplant (3 days)	4.89	5.63––5.96 (10––495)	Moorea, French Polynesia	Lin and Fong (2008)
Shrimp farm	––	*Gracilaria vermiculophylla*	Native	––	7.8––8.0 (––)[†d]	Gulf of California, Mexico	Piñón-Gimate et al. (2009)
Shrimp farm	––	*Hypnea spinella*	Native	––	8.5––11.4 (––)[†d]	Gulf of California, Mexico	Piñón-Gimate et al. (2009)
Shrimp farm	––	*Spyridia filamentosa*	Native	––	7.7 (––)[†d]	Gulf of California, Mexico	Piñón-Gimate et al. (2009)
Dicentrarchus labrax, *Sparus aurata*, *Diplodus puntazzo*[c]	300	*Sphaerococcus coronopifolius*	Native	––	5.8±1.7 (-)(–)	Sicily, Italy	Vizzini and Mazzola (2004)
Dicentrarchus labrax, *Sparus aurata*, *Diplodus puntazzo*[c]	300	*Padina pavonica*	Native	––	6.3±1.2 (-)(–)	Sicily, Italy	Vizzini and Mazzola (2004)

– unknown

[a] Shrimp farm

[b] Fish cages

[c] Land -based marine fish farms

[†d] The locations are influenced by various types of effluent, - unknown

Montoya 2009), and that in the food used on the farms studied by Vizzini and Mazzola (2004) varied between 6.2 ‰ and 11 ‰, according to Sarà et al. (2004).

The degree of effluent detection varied between the farms under study, as a result of differences in production (which determined the amount of dumping) and location (since sites presented different dispersive capacity). The waste from farm I is dumped in a harbour (Fig. 1) where there was a relatively low rate of hydric renovation, so the $\delta^{15}N$ was not less than 8 ‰ in any of the locations within the harbour. In regard to the farms located in zones where there was a high degree of hydrodynamism, the higher the production, the larger the area of influence (Table 1). Thus, the area of influence of farm IV with an annual production of 2,250 t (Table 1) was 800 m (site F), whereas for farm III (308 $tyear^{-1}$) and farm VI (43 $tyear^{-1}$), which dumped their waste in similar environments, the area of influence was less than 200 m in the predominating direction of the current.

Interestingly, the location most enriched in $\delta^{15}N$ did not correspond to those located closest to the focal point of dumping from these LBMFFs (site A, discharge point, Table 1), contrary to the results from similar studies of macroalgal $\delta^{15}N$ values in the vicinity of aquaculture farms (Costanzo et al. 2004; Jones et al. 2001; Lin and Fong 2008; Vizzini and Mazzola 2004). In these location, the $\delta^{15}N$ was impoverished, and it was even below the control site value in all of the farms under study (0.08–5.1 ‰ in *C. tomentosum* and 1.27–6.37 ‰ in *Fucus* sp., Table 1). These results agree with those of Rogers (1999) where impoverishment of $\delta^{15}N$ values in *Ulva lactuca* was observed adjacent to an urban waste dump, where the $\delta^{15}N$ decreased from 7.8 ‰ at the reference site to 3.4 ‰ in the location closest to the dumping point. This was associated with the particulate organic matter (POM), for which the $\delta^{15}N$ was similar (3.2 ‰), but which did not represent the fraction of N available to macroalgae. Another hypothesis can be that fractionation (preferential use of ^{14}N over ^{15}N) may occur during assimilation of nitrogen in primary producers when excess nitrogen is available for uptake. However, this seemed to be only true for phytoplankton (see e.g. Pennock et al. 1996), where lower $\delta^{15}N$ values were observed in producers relative to their source. Contrarily, different laboratory-based studies demonstrated that the $\delta^{15}N$ values of macroalgae accurately reflected nitrogen from water sources, even at high concentrations (Cohen and Fong 2005; Naldi and Wheeler 2002). Finally, the exceptionally low $\delta^{15}N$ values from the sites closest to the output may be related to pH. The mean pH of the input water was 8.03±0.03 and that of the output water was 7.81± 0.06 (average value of 68 samples from the marine fish farms studied provided by the water quality monitoring agency, Aguas de Galicia; Carballeira et al. 2012). Another possible explanation for the lower $\delta^{15}N$ values at the point of discharge and subsequently higher values along the coast is that the majority of the N in the effluent is ammonium. Macroalgae incorporate seven times more NH_4^+ than NO_3^- (Deutsch and Voss 2006) through passive diffusion (Vallyathan et al. 2002). The oxidation of NH_4^+ to NO_3^- increases $\delta^{15}N$ values (Hadas et al. 2009). Therefore, as the ammonium-rich effluent moves away from the point of discharge, more and more ammonium is oxidized to NO_3^-, and the $\delta^{15}N$ values subsequently increase.

It must therefore be taken into account that the determination of $\delta^{15}N$ values of macroalgae sampled at only one location located close to the dumping point may not adequately reflect the influence of the marine fish farm, and may lead to erroneous conclusions. In this way, the $\delta^{15}N$ for detecting LBMFFs effluents should be studied following a non-linear gradient, starting at the discharge point of the LBMFF effluent and following the direction of the prevailing current (Carballeira et al. 2012; Lapointe et al. 2007). Reference sites may be located at the opposite direction of the prevailing current, where most LBMFFs pump the input water, which must be clean. However, when cost is a limiting factor, a single location can be used to standardize the area of exposure. This measure requires the establishment of a maximum intra-annual $\delta^{15}N$ threshold value that should not be surpassed at a standard distance. For this type of LBMFFs and results, the standard distance must be more than 200 m from the waste disposal point, to ensure oxidation of the ammonium and re-equilibrium of the pH of the outgoing water with the environment so that it does not inhibit absorption of the N emitted.

The results from this study showed that the $\delta^{15}N$ values of macroalgae acted as descriptor of exposure, an indicator of the interaction between the loading estimate and the dispersive capacity of the environment, providing accurate information about the degree of contamination and the area of influence of a farm (Carballeira et al. 2011). However, despite the suitability of using native macroalgal $\delta^{15}N$ values to trace fish farming activities, certain aspects must be addressed before any plan for monitoring this type of industry can be implemented. These include (1) macroalga interspecific differences, (2) determination of the regional reference ranges of $\delta^{15}N$ and (3) intra-annual variability of $\delta^{15}N$.

Macroalgal interspecific differences

As different biomonitors are sometimes used at different sites, it was essential to determine whether there are any interspecific differences in the $\delta^{15}N$ values amongst species. Moreover, this may be necessary because of the tendency for shifts from perennial, native populations in areas enriched with nutrients, to opportunist macroalgae (Tett et al. 2007), which may lead to the need to substitute the species selected as a primary biomonitor. Umezawa et al. (2002) suggested that macroalgae that grow with the same inputs of nutrients (at least between taxonomically related

species) have similar values of $\delta^{15}N$. However, Deutsch and Voss (2006) observed large differences in the $\delta^{15}N$ values of *Enteromorpha* sp. and *Ulva* sp. even at the same stations. Although several authors have used different macroalgae as biomonitors of $\delta^{15}N$ (Gartner et al. 2002; Piñón-Gimate et al. 2009; Riera 1998; Tucker et al. 1999), only one quantitative comparison between the different types of macroalgae has been carried out until now (Dailer et al. 2010). In the present study, no significant differences in $\delta^{15}N$ were observed between the species that co-occur at the same location. For comparison between *F. vesiculosus* and *F. serratus*, the occurrence of significant differences between slopes (Fig. 2) was attributed to the small range of $\delta^{15}N$ values, so the slope could not be estimated accurately. This reflected a problem in the distribution of the data rather than real differences between the two species.

Determination of regional $\delta^{15}N$ values

Knowledge of the regional reference levels of $\delta^{15}N$ values of macroalgae may help to provide a more objective interpretation of the results obtained in local studies. The $\delta^{15}N$ average value from marine macroalgae worldwide is 7 ‰ (±4 ‰; Owens et al. 1988; Wada et al. 1975). Natural values from different species of macroalgae in different estuarine and marine environments range between 0.01 ‰ and 7.3 ‰ (Burford et al. 2003; Dailer et al. 2010; Gartner et al. 2002; Jones et al. 2001; Lin and Fong 2008; Rogers 2003).

Because of the large natural variation in $\delta^{15}N$ values of macroalgae, it appears that the reference range must be established for each species in a particular region. The regional reference range in the study area was 5.48±1.18 ‰ (calculated from the ESBG 2007 survey data, Fig. 3). Taking into account the distribution of values, it was found that the location furthest from the dumping point (sites D to H, Table 1) were within the established regional reference range, and therefore outside the area of influence of the farms. These locations defined the first modal value observed in the distribution of all the locations sampled in the surroundings of marine fish farms (Fig 3), and which were almost consistent with the modal value of the distribution of the regional reference values. The second modal value, which was higher than the reference range, corresponded to sites that were apparently more influenced by dumping from the marine fish farms. The left tail of the distribution, with values of $\delta^{15}N$ lower than reference values, was formed by the sites closest to the waste emission points (site A, Table 1). The wide fluctuation in the $\delta^{15}N$ signal in macroalgae, from 5.48 ‰ (control) to 11.7 ‰ (maximum value observed), enabled better identification of the degree of exposure to the contaminants relative to that in other organisms showing narrower ranges of variations (Lamb et al. 2012).

Intra-annual variability

Macroalgal $\delta^{15}N$ values obtained at one particular time of year may not be representative of the entire year because of the potential annual variability in the $\delta^{15}N$ values of the source effluent. Although at the control site, the mean $\delta^{15}N$ value was approximately 6.29±1.19 ‰ throughout the year (Fig. 4), the $\delta^{15}N$ of macroalgae sampled at the sites affected by disposal from the marine fish farms varied by 5.57 ‰ (4.67 ‰ to 10.24 ‰), the highest values being observed during summer, when the highest temperatures were recorded, and in consequence, the greatest rates of fish production were expected. However, the maximum annual value for the control was not synchronous with the maximum value for any of the locations affected by the marine fish farms.

In conclusion, the present study has shown that macroalgal $\delta^{15}N$ values may be an excellent way of monitoring exposure to organic wastes from marine fish farm effluents. This parameter integrated environmental conditions and provided information about the bioavailable fraction of nitrogen. It was easy to interpret and highly replicable. Furthermore, macroalgal $\delta^{15}N$ values determined the area of influence and the variation of influence over time. The results obtained also showed that there were no significant differences in $\delta^{15}N$ values measured in the species *Fucus* spp. and *C. tomentosum*; therefore, these macroalgae can be used indistinctly as biomonitors.

However, for future studies, correlations should be established between the changes in the descriptor of exposure ($\delta^{15}N$), and direct or indirect changes in the composition or functioning of the affected coastal ecosystems in order to evaluate whether $\delta^{15}N$ values can be used to predict environmental deterioration. This way, environmental monitoring could be performed by using this simple, cheap and rapid tool in replacement of more complex measurements.

Acknowledgments The present study was partly financed by the Spanish Government's National Plan for Marine Culture (JACUMAR, 2008): "Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)". Carlos Carballeira is grateful to the University of Cadiz Predoctoral Fellowships Programme (Spain).

References

APROMAR (2011) La acuicultura marina en España. APROMAR. www.apromar.es/Informes/. Accessed 21 November 2011

Burford MA, Costanzo SD, Dennison WC, Jackson CJ, Jones AB, McKinnon AD, Preston NP, Trott LA (2003) A synthesis of dominant ecological processes in intensive shrimp ponds and adjacent coastal environments in NE Australia. Mar Pollut Bull 46:1456–1469

Carballeira A, Carral E, Puente X, Villares R (2000) Regional-scale monitoring of coastal contamination. Nutrients and heavy metals in estuarine sediments and organisms on the coast of Galicia (northwest Spain). Int J Environ Pollut 13:534–572

Carballeira C, Espinosa J, Carballeira A (2011) Linking $\delta^{15}N$ and histopathological effects in molluscs exposed in situ to effluents from land-based marine fish farms. Mar Pollut Bull 62:2633–2641

Carballeira C, Ramos-Gómez J, Martín-Díaz ML, DelValls TA, Carballeira A (2012) Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas. J Environ Monit. doi:10.1039/c2em10839a

Cloern JE (2001) Our evolving conceptual model of the coastal eutrophication problem. Mar Ecol Prog Ser 210:223–253

Cohen RA, Fong P (2005) Experimental evidence supports the use of $\delta^{15}N$ content of the opportunistic green macroalga *Enteromorpha intestinalis* (Chlorophyta) to determine nitrogen sources to estuaries. J Phycol 41:287–293

Costanzo SD, O'Donohue MJ, Dennison WC, Loneragan NR, Thomas M (2001) A new approach for detecting and mapping sewage impacts. Mar Pollut Bull 42:149–156

Costanzo SD, O'Donohue MJ, Dennison WC (2004) Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. Mar Pollut Bull 48:514–525

Dailer ML, Knox RS, Smith JE, Napier M, Smith CM (2010) Using $\delta^{15}N$ values in algal tissue to map locations and potential sources of anthropogenic nutrient inputs on the island of Maui, Hawaii, USA. Mar Pollut Bull 60:655–671

Dailer ML, Ramey HL, Saephan S, Smith CM (2012) Algal $\delta^{15}N$ values detect a wastewater effluent plume in nearshore and offshore surface waters and three-dimensionally model the plume across a coral reef on Maui, Hawaii, USA. Mar Pollut Bull. doi:10.1016/j.marpolbul.2011.12.004

Dalsgaard T, Krause-Jensen D (2006) Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. Aquaculture 256:302–310

Deutsch B, Voss M (2006) Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: results from in-situ incubation experiments. Sci Total Environ 366:799–808

Dolenec T, Lojen S, Lambasa S, Dolenec M (2006) Effects of fish farm loading on sea grass *Posidonia oceanica* at Vrgada Island (Central Adriatic): a nitrogen stable isotope study. Isot Environ Health Stud 42:77–85

Ervik A, Hansen PK, Aure J, Stigebrandt A, Johannessen P, Jahnsen T (1997) Regulating the local environmental impact of intensive marine fish farming I. The concept of the MOM system. Aquaculture 158:85–94

FAO (2010) The state of world fisheries and aquaculture. Food and Agricultural Organization of the United Nations, Rome

Fernandes TF, Eleftheriou A, Ackefors H, Eleftheriou M, Ervik A, Sanchez-Mata A, Scanlon T, White P, Cochrane S, Pearson TH, Read PA (2001) The scientific principles underlying the monitoring of the environmental impacts of aquaculture. J Appl Ichthyol 17:181–193

Filgueira R, Castro BG (2011) Study of the trophic web of San Simón Bay (Ría de Vigo) by using stable isotopes. Cont Shelf Res 31:476–487

García-Sanz T, Ruiz-Fernández JM, Ruiz M, García R, González MN, Pérez M (2010) An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. Mar Environ Res 70:189–200

Gartner A, Lavery P, Smit AJ (2002) Use of $\delta^{15}N$ signatures of different functional forms of macroalgae and filter-feeders to reveal temporal and spatial patterns in sewage dispersal. Mar Ecol Prog Ser 235:63–73

Hadas O, Altabet MA, Agnihotri R (2009) Seasonally varying nitrogen isotope biogeochemistry of particulate organic matter in Lake Kinneret, Israel. Limnol Oceanogr 54:75–85

Heaton THE (1986) Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. Isot Geosci 59:87–102

Hoering T (1955) Variations of nitrogen^{15} abundance in naturally occurring substances. Science 122:1233–1234

Jones AB, O'Donohue MJ, Udy J, Dennison WC (2001) Assessing ecological impacts of shrimp and sewage effluent: biological indicators with standard water quality analyses. Estuar Coast Shelf Sci 52:91–109

Lamb K, Swart PK, Altabet MA (2012) Nitrogen and carbon isotopic systematics of the Florida reef tract. Bull Mar Sci 88:119–146

Landrum JP, Montoya JP (2009) Organic matter processing by the shrimp *Palaemonetes* sp.: isotopic and elemental effects. J Exp Mar Biol Ecol 380:20–24

Lapointe BE, Bedford BJ (2007) Drift rhodophyte blooms emerge in Lee County, Florida, USA: evidence of escalating coastal eutrophication. Harmful Algae 6:421–437

Lapointe BE, Langton R, Bedford BJ, Potts AC, Day O, Hu C (2010) Land-based nutrient enrichment of the Buccoo Reef Complex and fringing coral reefs of Tobago, West Indies. Mar Pollut Bull 60:334–343

Lin DT, Fong P (2008) Macroalgal bioindicators (growth, tissue N, $\delta^{15}N$) detect nutrient enrichment from shrimp farm effluent entering Opunohu Bay, Moorea, French Polynesia. Mar Pollut Bull 56:245–249

Lobban CS, Harrison PJ (1994) Seaweed ecology and physiology. Cambridge University Press, Cambridge

Lojen S, Spanier E, Tsemel A, Katz T, Eden N, Angel D (2005) $\delta^{15}N$ as a natural tracer of particulate nitrogen effluents released from marine aquaculture. Mar Biol 148:87–96

Macko SA, Ostrom NE (1994) Pollution studies using stable isotopes. In: Michener R, Lajtha K (eds) Stable isotopes in ecology and environmental science. Blackwell Scientific, London, pp 42–65

Mattern S, Sebilo M, Vanclooster M (2011) Identification of the nitrate contamination sources of the Brusselian sands groundwater body (Belgium) using a dual-isotope approach. Isot Environ Health Stud 47:297–315

McClelland JW, Valiela I, Michener RH (1997) Nitrogen-stable isotope signatures in estuarine food webs: a record of increasing urbanization in coastal watersheds. Limnol Oceanogr 42:930–937

Michener R, Lajtha K (2007) Stable isotopes in ecology and environmental science, 2nd edn. Blackwell Publishing, Malden

Minagawa M, Wada E (1984) Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. Geochim Cosmochim Acta 48:1135–1140

Miyake Y, Wada E (1967) The abundance ratio of $^{15}N/^{14}N$ in marine environments. Rec Oceanogr Works Jpn 9:37–53

Naldi M, Wheeler PA (2002) ^{15}N Measurements of ammonium and nitrate uptake by *Ulva fenestrata* (chlorophyta) and *Gracilaria pacifica* (rhodophyta): comparison of net nutrient disappearance, release of ammonium and nitrate, and ^{15}N accumulation in algal tissue. J Phycol 38:135–144

Nier AO (1950) A redetermination of the relative abundances of the isotopes of neon, krypton, rubidium, xenon, and mercury. Phys Rev 79:450–454

Owens NJP, Blaxter JHS, Southward AJ (1988) Natural variations in ^{15}N in the marine environment. Adv Mar Biol 24:389–451

Pennock JR, Velinsky DJ, Ludlam JM, Sharp JH, Fogel ML (1996) Isotopic fractionation of ammonium and nitrate during uptake by *Skeletonema costatum*: implications for $\delta^{15}N$ dynamics under bloom conditions. Limnol Oceanogr 41:451–459

Piñón-Gimate A, Soto-Jimenez MF, Ochoa-Izaguirre M, García-Pages E, Paez-Osuna F (2009) Macroalgae blooms and $\delta^{15}N$ in subtropical coastal lagoons from the Southeastern Gulf of California: discrimination among agricultural, shrimp farm and sewage effluents. Mar Pollut Bull 58:1144–1151

R Development Core Team (2008) R: a language and environment for statistical computing, vol 1. R Foundation for Statistical Computing, Vienna, p 7

Read P, Fernandes T (2003) Management of environmental impacts of marine aquaculture in Europe. Aquaculture 226:139–163

Riera P (1998) $\delta^{15}N$ of organic matter sources and benthic invertebrates along an estuarine gradient in Marennes-Oleron Bay

(France): implications for the study of trophic structure. Mar Ecol Prog Ser 166:143–150
Riera P, Stal LJ, Nieuwenhuize J (2000) Heavy $\delta^{15}N$ in intertidal benthic algae and invertebrates in the Scheldt Estuary (The Netherlands): effect of river nitrogen inputs. Estuar Coast Shelf Sci 51:365–372
Robinson D (2001) $\delta^{15}N$ as an integrator of the nitrogen cycle. Trends Ecol Evol 16:153–162
Rogers KM (1999) Effects of sewage contamination on macro-algae and shellfish at Moa Point, New Zealand using stable carbon and nitrogen isotopes. NZ J Mar Freshw Res 33:181–188
Rogers KM (2003) Stable carbon and nitrogen isotope signatures indicate recovery of marine biota from sewage pollution at Moa Point, New Zealand. Mar Pollut Bull 46:821–827
Sarà G, Scilipoti D, Mazzola A, Modica A (2004) Effects of fish farming waste to sedimentary and particulate organic matter in a southern Mediterranean area (Gulf of Castellammare, Sicily): a multiple stable isotope study ($\delta^{13}C$ and $\delta^{15}N$). Aquaculture 234:199–213
Savage C (2005) Tracing the influence of sewage nitrogen in a coastal ecosystem using stable nitrogen isotopes. Ambio 34:145–150
Savage C, Elmgren R (2004) Macroalgal (*Fucus vesiculosus*) $\delta^{15}N$ values trace decrease in sewage influence. Ecol Appl 14:517–526
Struck U (2012) On the use of stable nitrogen isotopes in present and past anoxic environments anoxia. In: Altenbach AV, Bernhard JM, Seckbach J (eds) Cellular origin, life in extreme habitats and astrobiology, vol 21. Springer, Dordrecht, pp 497–513
Tello A, Corner RA, Telfer TC (2010) How do land-based salmonid farms affect stream ecology? Environ Pollut 158:1147–1158
Tett P, Gowen R, Mills D, Fernandes T, Gilpin L, Huxham M, Kennington K, Read P, Service M, Wilkinson M, Malcolm S (2007) Defining and detecting undesirable disturbance in the context of marine eutrophication. Mar Pollut Bull 55:282–297
Tucker J, Sheats N, Giblin AE, Hopkinson CS, Montoya JP (1999) Using stable isotopes to trace sewage-derived material through Boston Harbor and Massachusetts Bay. Mar Environ Res 48:353–375
Umezawa Y, Miyajima T, Yamamuro M, Kayanne H, Koike I (2002) Fine-scale mapping of land-derived nitrogen in coral reefs by $\delta^{15}N$ in macroalgae. Limnol Oceanogr 47:1405–1416
Vallyathan V, Castranova V, Shi X (2002) Oxygen/nitrogen radicals: Cell injury and disease, vol 234/235. Kluwer Academic, Massachusetts
Van Dover CL, Grassle JF, Fry B, Garritt RH, Starczak VR (1992) Stable isotope evidence for entry of sewage-derived organic material into a deep-sea food web. Nature 360:153–156
Viana IG, Aboal JR, Fernández JA, Real C, Villares R, Carballeira A (2010) Use of macroalgae stored in an Environmental Specimen Bank for application of some European Framework Directives. Water Res 44:1713–1724
Viana IG, Fernández JA, Aboal JR, Carballeira A (2011) Measurement of $\delta^{15}N$ in macroalgae stored in an environmental specimen bank for regional scale monitoring of eutrophication in coastal areas. Ecol Indic 11:888–895
Villares R, Carballeira A (2003) Seasonal variation in the concentrations of nutrients in two green macroalgae and nutrient levels in sediments in the Rías Baixas (NW Spain). Estuar Coast Shelf Sci 58:887–900
Villares R, Carballeira A (2004) Nutrient limitation in macroalgae (*Ulva* and *Enteromorpha*) from the Rías Baixas (NW Spain). Mar Ecol 25:225–243
Villares R, Carballeira A (2006) Trophic categorization in the Rías Baixas (NW Spain): nutrients in water and in macroalgae. Sci Mar 70(1):89–97
Vizzini S, Mazzola A (2004) Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Mar Pollut Bull 49:61–70
Vosz M, Struck U (1997) Stable nitrogen and carbon isotopes as indicator of eutrophication of the Oder River (Baltic Sea). Mar Chem 59:35–49
Wada E, Hattori A (1976) Natural abundance of ^{15}N in particulate organic matter in the North Pacific Ocean. Geochim Cosmochim Acta 40:249–251
Wada E, Kadonaga T, Matsuo S (1975) ^{15}N abundance in nitrogen of naturally occurring substances and global assessment of denitrification from isotopic viewpoint. Geochem J 9:139–148
Warton D, Ormerod J (2007) Smatr: Standardised Major Axis Estimation and Testing Routines. R package, 2.1 edn. http://web.maths.unsw.edu.au/~dwarton
Wolanski E, Spagnol S, Thomas S, Moore K, Alongi DM, Trott L, Davidson A (2000) Modelling and visualizing the fate of shrimp pond effluent in a mangrove-fringed tidal creek. Estuar Coast Shelf Sci 50:85–97
Yang X, Wu X, Hao H, He Z (2008) Mechanisms and assessment of water eutrophication. J Zhejiang Univ 9(3):197–209

Interannual changes in $\delta^{15}N$ values in *Fucus vesiculosus* L.

I. G. Viana[1,3], C. Carballeira[2], A. Rey-Asensio[1], A. Carballeira[1]

[1]Área de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, Spain.
[2]UNITWIN/UNESCO/WiCoP. Physical Chemistry Department, CASEM, Universidad de Cádiz,11510, Puerto Real. Cádiz. Spain.
[3]Present address: Instituto Español de Oceanografía, Centro Costero de A Coruña, Apdo. 130, E-15080, A Coruña, Spain. e-mail: ines.gonzalez@co.ieo.es

Abstract

The $\delta^{15}N$ has been widely used to detect anthropogenic loads in numerous environmental impact studies. A dendroanalysis was carried out during a period of three years (2008-2010) in subsamples of *Fucus vesiculosus* L. growing at two sites: a control site, within a coastal reference area, and an area affected by the effluents of a land-based marine fish farm. The results show that isotopic values from different parts (or growing moments) of macroalgae vary depending on the production load. Moreover, the isotopic signal decreased with the age of the frond to a certain range of values. The $\delta^{15}N$ signal from *F. vesiculosus* is temporally unstable; therefore, isotopic signal $\delta^{15}N$ does not allow reliable retrospective biomonitoring of environmental pollution. Further knowledge about the growth and other biological aspects of this species is required.

Keywords: dendroanalysis; stable isotopes; nitrogen; biomonitoring; macroalgae.

1. Introduction

Stable isotope analysis (SIA) allows tracing contamination, in either biotic or abiotic matrices (Dolenec et al., 2006; Oevelen et al., 2007; Sarà et al., 2006). The ratio of stable nitrogen isotopes ($\delta^{15}N$) has been increasingly used in the last years to quantify the extent of eutrophication brought about by N loading (Bode et al., 2011; Conlan et al., 2006; Lapointe et al., 2010). The method is based on the fact that N has two stable isotopes, a light isotope, ^{14}N, and a heavier isotope, ^{15}N, which occur in a constant proportion in the atmosphere (Nier, 1950). The different anthropogenic sources of N alter the baseline levels of $\delta^{15}N$ in marine systems (Heaton, 1986), so it can be used to trace and quantify different terrestrial inputs.

Several species may be chosen as biomonitors by determining the changes in the isotopic values in their tissues (Jones et al., 2001; Riera et al., 2000; Savage, 2005; Tewfik et al., 2005; Vizzini and Mazzola, 2004). Macroalgae were selected in various environmental studies (Costanzo et al., 2001; Dailer et al., 2012; McClelland and Valiela, 1998) because they are good biomonitors of contamination for several reasons: i) they absorb the dissolved inorganic nitrogen (DIN) of water and accumulate it in their tissues, ii) macroalgae temporally integrates the ^{15}N source signal because of the slow rate of turnover of their tissues (Lobban and Harrison, 1994; McClelland et al., 1997; Voβ and Struck, 1997), iii) $\delta^{15}N$ in macroalgae is temporally stable under natural conditions (Savage and Elmgren, 2004; Carballeira et al., 2012), iv) the range of variation of the isotopic values in the macroalgae is higher than other organisms (Riera et al., 2000), this sensitivity enables better discrimination of N sources, and v) macroalgae are abundant, easy to sample and handle.

Among macroalgae species, the genus *Fucus* is characterized by their apical growth (Knight and Parke, 1950). This fact enables to relate and standardize a subsample of the individual (e.g. apical tip) with a particular exposure time. Savage and Elmgren (2004) first used this characteristic to subsample *Fucus vesiculosus* specimens to determine the temporal changes in isotopic composition within individual plants and to subsequently determine the efficiency of upgrading a water treatment plant. Therefore, macroalgae with apical growing may be used in retrospective contamination studies. This dendrochemical-type approach may enable obtaining information about environmental issues from the past (Hagemeyer, 1995). The use of this approach may simplify the periodicity of sampling surveys from biomonitoring studies, thus reducing the costs, particularly those associated with regional scale studies (Viana et al., 2011).

Macroalgae are exposed to shifts in the $\delta^{15}N$ of the DIN, and their tissues may reflect those shifts (Savage and Elmgren, 2004). Nevertheless, in order to perform a reliable dendrochemical approach, it is necessary to verify if the $\delta^{15}N$ is stable over time within algal tissues (Nabais et al., 1999). For this purpose, each specimen must be subsampled, according to the length and frond dichotomies, to provide an approximate representation of previous

years (Savage and Elmgren, 2004) and to determine temporal changes in isotopic composition. The aim of the present study was to verify whether the $\delta^{15}N$ in *F. vesiculosus* L. is stable, in order to ensure feasible interannual retrospective studies.

2. Material and methods

The study was carried out in two intertidal areas on the coast of Galicia, NW Spain (Fig. 1). Sampling was carried out in August of three consecutive years:

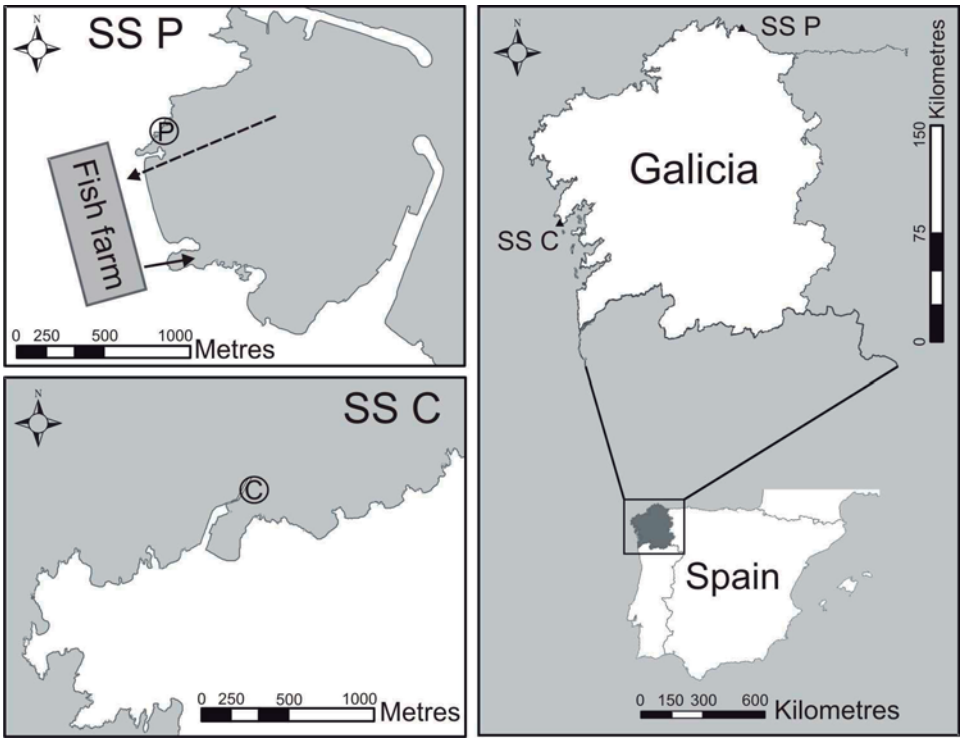


Figure 1. On the right panel there is a map showing the location of Galicia in NW Spain and the locations of the sampling sites (solid triangles). Enlarged top left box shows the surroundings of the land-based fish farm and the location of SS P, influenced by fish farm loadings. The dashed arrow indicates the water input and the solid black arrow indicates the discharge output of farms. Enlarged bottom left box shows the location of SS C in a reference area.

2008, 2009 and 2010. One sampling site (SS) is affected by nitrogen enriched inputs from a marine land-based fish farm located at Xove (SS P). This fish farm grows flatfish, basically turbot (*Psetta maxima*) and the impact of its effluents on the isotopic signal $\delta^{15}N$ of several macroalgal specieswas first studied by Carballeira et al. (2012). The mean production on this farm fluctuated from 500 in 2005 to 2250 t $year^{-1}$ of turbot in 2008.

The other SS is a reference site at Lira, SS C (Viana et al., 2011). At each SS, three fixed to the substrate individuals of *F. vesiculosus* were collected in the mesolittoral zone. These specimens were washed with seawater to remove particulate material and transported to the laboratory where epiphytes were also removed, and washed again with bidistilled water. The samples were frozen (-28 ± 2 °C) until they were processed.

Specimens were subsampled attending to the frond length and the number of dichotomies. These subsamples were separated from the rest of the plant with a glass spatula. Samples were then dried at 45 °C in a forced air oven and homogenized in an ultracentrifugal mill (Retsch ZM 100). Dried samples were then stored at room temperature in glass vessels.

Stable isotope analysis was carried out by the Unidad de Técnicas Instrumentales de Análisis, University of A Coruña following the same procedure as the one described in Viana et al. (2011). The relative abundance of ^{15}N in the sample ($\delta^{15}N$) was calculated from the formula: $\delta^{15}N$ (‰) = $[(R_{sample}/R_{standard})-1]*10^3$, where R is the $^{15}N/^{14}N$ ratio. The overall error was determined by use of analytical replicates. This constitutes a measure of precision as it is the coefficient between the standard deviation of the replicates and the number of replicates. The overall error for the replicates was ≤ 2%.

One-way ANOVA was used to find any differences in the $\delta^{15}N$ of the subsamples from individual specimens of the same cohort (developed at the same time) but subsampled in different

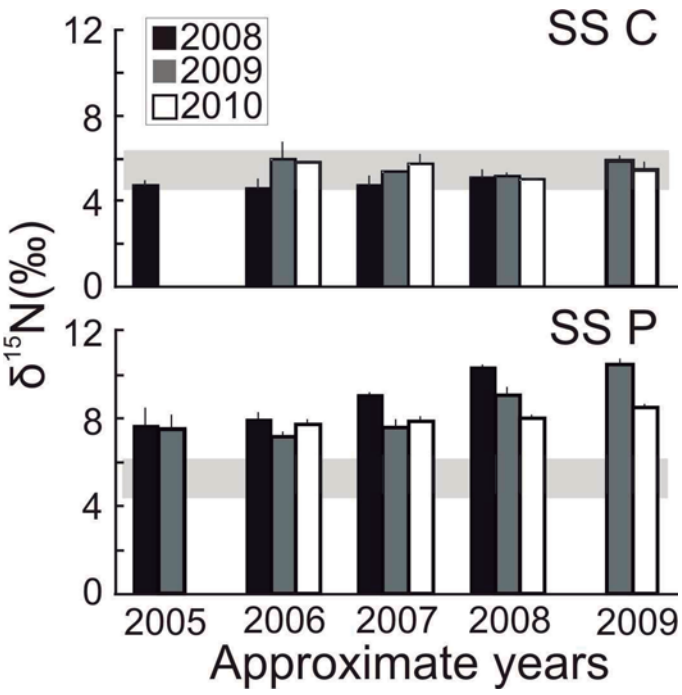


Figure 2. Temporal variance in the $\delta^{15}N$ values (‰ mean±SE) from the subsamples of *F. vesiculosus* collected at the coastal reference area (SS C) and the area affected by land-based marine fish farm effluents (SS P). Different colours of bars represent the three different sampling years: 2008 (black), 2009 (grey) and 2010 (white). The grey band represents the regional reference range (5.48±1.18‰, Viana et al., 2011).

years. The above mentioned tests were carried out with PASW Statistics 18.

3. Results and discussion

The $\delta^{15}N$ values in macroalgal subsamples collected at the two SS in the years 2008, 2009 and 2010 are shown in Table 1.

Interannual changes of $\delta^{15}N$ values are represented in Fig. 2. There were no significant differences ($p< 0.01$) between the values for each cohort at SS C (Table 1), besides, all values were within the range of the regional reference values, 5.48±1.18‰ (Fig. 3)(Viana et al., 2011). In contrast, all cohorts from macroalgae collected at SS P showed $\delta^{15}N$ values above the natural regional range (Fig. 3). These enriched values may be caused, as expected, by the chronic influence of the N loadings from the fish farm, as it was initially detected by Carballeira et al. (2012).

Macroalgae sampled at the SS C did not show significant differences between subsamples of different sampling years and same cohort. In contrast, isotopic values from macroalgae in SS P showed significant differences ($p<0.01$) between the subsamples of the same cohort, sampled at different years, i.e. between the parts of the fronds that represent the same year.

Table 1. $\delta^{15}N$ values (‰ mean±SD) from three individual subsamples of *F. vesiculosus* collected (2008-2010) at the coastal reference area (SS C) and at the area affected by a land-based marine fish farm effluents (SS P). (ANOVA – Differences between cohorts: ns = not significant; ** $p<0.01$; *** $p<0.001$).

		Sampling years				
SS	Cohort	2008	2009	2010	F	*p*
C	2005	4.76±0.20	-	-		
	2006	4.61±0.41	5.97±0.78	5.85±0.04	5.44	ns
	2007	4.75±0.43	5.36±0.10	5.73±0.48	1.71	ns
	2008	5.09±0.40	5.17±0.17	5.00±0.01	0.12	ns
	2009	-	5.92±0.17	5.47±0.35	135	ns
	2010	-	-	6.77±0.49		
P	2005	7.67±0.82	7.50±0.65	-	0.26	ns
	2006	7.93±0.34	7.18±0.17	7.70±0.24	2.52	ns
	2007	9.06±0.12	7.56±0.34	7.83±0.24	10.3	**
	2008	10.28±0.20	9.02±0.35	7.97±0.19	20.62	***
	2009	-	10.47±0.18	8.47±0.20	53.67	***
	2010	-	-	9.27±0.27		

These results confirm the temporal stability of the $\delta^{15}N$ in *F. vesiculosus* from the SS C at an interannual scale. They confirm that macroalgae at this site have had constant and low anthropogenic nitrogen influence during the sampling years. In contrast, the $\delta^{15}N$ in the subsamples of the same cohort of macroalgae at SS P are significantly different during the sampling years ($p>0.05$). Moreover, $\delta^{15}N$ values from cohorts decreased significantly with the age of the frond. The enriched $\delta^{15}N$ values observed in the sampling from 2008 tend to decrease until a certain range (7-8‰), which is still higher than reference values. Even though, the output from the fish farm was continuous during the years of the study, the intensity of this waste may have varied, as a consequence of the increasing production (Table 1). During these years, macroalgae have been influenced by different intensity loadings which is reflected in the different subsamples of the individuals sampled at the same year (Table 1). But when subsamples of the same cohort but different sampling years are compared, significant differences were found (Table 1). The decrease in the isotopic ratio indicates a reduction of the concentration of ^{15}N in comparison with that of ^{14}N which may be explained by:

i) The cellular location of the N may depend on the source of the ^{15}N isotope; therefore, some intracellular fractions may display more conservative signals than extracellular fractions. If ^{15}N from particulate material is adhered externally, it may be more easily eliminated than ^{15}N absorbed from solution. (Costas and López, 2001).

ii) Thallusabsorb nutrients from the surrounding water but the nutrient content increases slower than thallus weight. In this manner, large thalli tend to have reduced nutrient levels (Geertz-Hansen et al., 1994).

iii) Growth rate of *F. vesiculosus* showed pronounced latitudinal differences (Mathieson et al., 1976). Knight and Parke (1950) found average rates of 1.9-2.7 cm $month^{-1}$ in Great Britain. In contrast, Fuentes (1986) observed somewhat lower rates of 0.6-1.8 cm $month^{-1}$ in sites close to the SS.

Considering that algal growth rate decreases with the age of the frond (Stevenson et al., 1996), we may assume that the isotopic signal in the subsamples collected at SS C in the last two years of the study may be

representative of a different period. However, all SS C values were within the same range, proving the natural stability of $\delta^{15}N$.

However, there is no clear explanation of the convergence of the $\delta^{15}N$ of the frond to a certain level. When values of the isotopic signal from the dendrochemical-type analysis of the samples collected in the last year (7.97-8.47-9.27‰, Table 1) are compared with background regional levels, then, these values are not natural. The high values of $\delta^{15}N$ observed in macroalgae from aquaculture impacted sites proved the influence of their effluents on the surroundings. However, there was a decline on $\delta^{15}N$ values with time, probably due to the elimination of externally adhered $\delta^{15}N$ (from particulate matter), the variability of environmental conditions or a decrease in the farm production rate. Further studies of the biology of this macroalgal species (i.e. on growth, absorption, and retention of dissolved N and isotopes) are required for standardizing its use as a standard biomonitor. As well as standardizing the time of exposure of *F. vesiculosus* for monitoring purposes by analyzing the parts of the frond that belong to the same growing period.

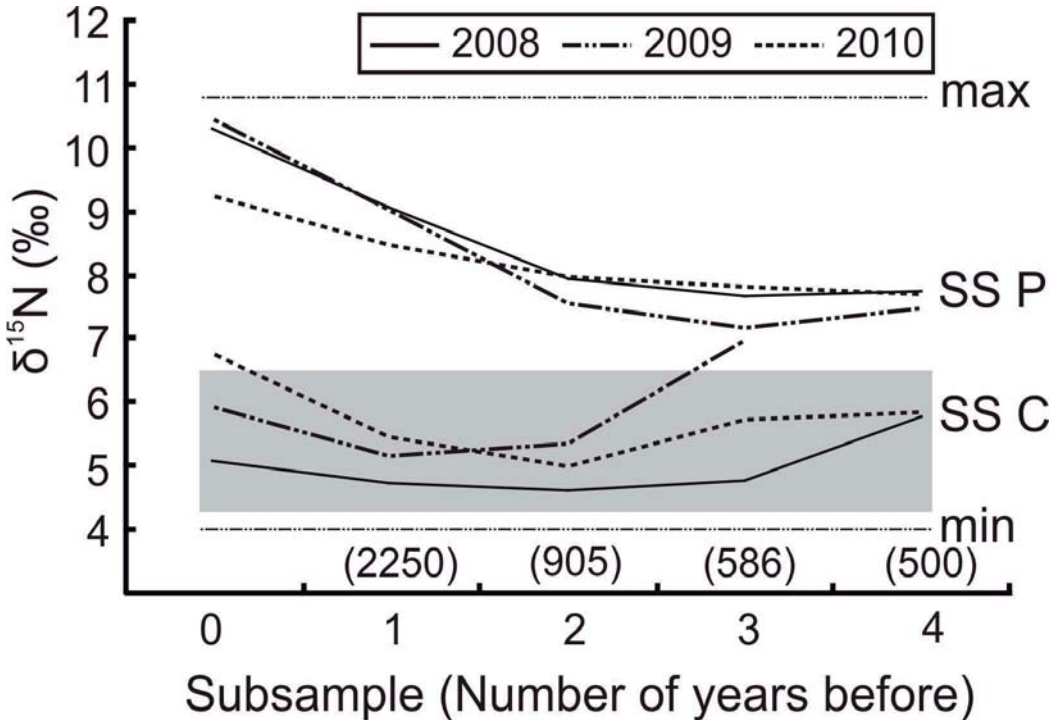


Figure 3. Trends of the isotopic signal $\delta^{15}N$ from samples of *F. vesiculosus* collected in the coastal reference area (SS C) and the area affected by a land-based marine fish farm (SS P). The grey band represents the regional reference range (5.48±1.18‰) and the max and min lines represent maximal and minimal regional values observed by Viana et al. (2011). The fish farm productions are indicated with brackets, in $t.year^{-1}$, for each subsample period.

Acknowledgements

This study was partly funded by the National Marine Aquaculture Plan. JACUMAR Project (2008): "Selection of Indicators, determination of reference values, design of programmes, protocols and Measures for environmental studies in aquaculture (INDAQUA)." Carlos Carballeira is grateful for funding from the University of Cádiz Predoctoral Fellowships Programme (Spain).

References

Bode, A., Varela, M., Prego, R., 2011. Continental and marine sources of organic matter and nitrogen for rías of northern Galicia (Spain). Marine Ecology Progress Series 437, 13-26.

Carballeira, C., Viana, I.G., Carballeira, A., 2012. $\delta^{15}N$ in macroalgae as an indicator of the potential impact of waste disposal from land-based marine fish farms. Journal of Applied Phycology, doi: 10.1007/s10811-012-9843-z.

Conlan, E.K., Kvitek, R.G., 2006. $\delta^{13}C$ and $\delta^{15}N$ shifts in benthic invertebrates exposed to sewage from McMurdo Station, Antarctica. Marine Pollution Bulletin 52, 1695-1707.

Costanzo, S.D., O´Donohue, M.J., Dennison, W.C., Loneragan, N.R., Thomas, M., 2001. A new approach for detecting and mapping sewage impacts. Marine Pollution Bulletin 42(2), 149-156.

Costas, R.C., López, J., 2001. Application of the sequential elution technique to determine Cd and Cu cellular location in *Ulva lactuca* Linnaeus. Archives of Environmental Contamination and Toxicology 41, 427-435.

Dailer, M.L., Ramey, H.L., Saephan, S., Smith, C.M., 2012. Algal $\delta^{15}N$ values detect a wastewater effluent plume in nearshore and offshore surface waters and three-dimensionally model the plume across a coral reef on Maui, Hawai'i, USA. Marine Pollution Bulletin 64, 207-213.

Deutsch, B., Voβ, M., 2006. Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: Results from in-situ incubation experiments. Science of the Total Environment 366, 799-808.

Dolenec, T., Lojen, S., Lambaša, S., Dolenec, M., 2006. Effects of fish farm loading on sea grass *Posidonia oceanica* at Vrgada Island (Central Adriatic): a nitrogen stable isotope study. Isotopes in Environmental and Health Studies 42(1), 77-85.

Fuentes, J.M., 1986. Dinámica, estructura y producción de una comunidad fitobentónica intermareal (horizonte de *Fucus vesiculosus*) en las Rías Gallegas. PhD Thesis, Universidad de Málaga.

Geertz-Hansenl, O, Enriquez, S., Duarte, C.M., Agusti, S., Vaqué, D., Vidondo, B., 1994. Functional implications of the form of *Codium bursa*, a balloon-like Mediterranean macroalga. Marine Ecology Progress Series 108, 153-160.

Hagemeyer, J., 1995. Radial distributions of Cd in stems of oak trees (*Quercus robur* L.) re-analyzed after 10 years. Trees 9, 200-203.

Heaton, T.H.E., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. Chemical Geology (Isotope Geoscience Section) 59, 87-102.

Jones, A.B., O´Donohue, M.J.O., Udy, J., Dennison, C., 2001. Assessing ecological impacts of shrimp and sewage effluent: Biological indicators with standard water quality analyses. Estuarine, Coastal and Shelf Science 52, 91-109.

Knight, M., Parke, M., 1950. A biological study of *Fucus vesiculosus* L. and *Fucus serratus* L. Journal of the Marine Biological Association of the United Kingdom 24, 439-515.

Lapointe, B. E., Langton, R., Bedford, B.J., Potts, A.C., Day, O., Hu, C., 2010. Land-based nutrient enrichment of the Buccoo Reef Complex and fringing coral reefs of Tobago, West Indies. Marine Pollution Bulletin. 60, 334-343.

Lobban, C.S., Harrison, P.J., 1994. Seaweed ecology and physiology. Cambridge University Press.

Mathieson, A.C., Shipman, J.W., O´Shea, J.R., Hasevlat, R.C., 1976. Seasonal growth and reproduction of estuarine fucoid algae in New England. Journal of Experimental Marine Biology and Ecology 25(3), 273-284.

McClelland, J.W., Valiela, I., 1998. Linking nitrogen in estuarine producers to land-derived sources. Limnology and Oceanography 43(4), 577-585.

McClelland, J.W., Valiela, I., Michener, R.H., 1997. Nitrogen-stable isotope signatures in estuarine food webs: A record of increasing urbanization in coastal watersheds. Limnology and Oceanography 42(5), 930-937.

Nabais, C., Freitas, H., Hagemeyer, J., 1999. Dendroanalysis: a tool for biomonitoring environmental pollution? The Science of the Total Environment 232(1-2), 33-37.

Nier, A.O., 1950. A redetermination of the relative abundances of the isotopes of carbon, nitrogen, oxygen, argon and potassium. Physics Review 77, 789-793.

Oevelen van D., Soetaert, K., Franci, M.A., Moodley, L., Ijzerloo, van L., Vincx, M., Vanaverbeke, J., 2007. Organic matter input and processing in two contrasting North Sea sediments: insights from stable isotope and biomass data. Marine Ecology Progress Series 380, 19-32.

Riera, P., Stal, L.J., Nieuwenhuize, J., 2000. Heavy $\delta^{15}N$ in intertidal benthic algae and invertebrates in the Scheldt Estuary (The Netherlands): Effect of River Nitrogen Inputs. Estuarine, Coastal and Shelf Science 51, 365-372.

Sarà, G. 2006. Hydrodynamic effects on the origin and quality of organic matter for bivalves: an integrated isotopic, biochemical and transplant study. Marine Ecology Progress Series 328, 65-73.

Savage, C., Elmgren, R., 2004. Macroalgal (*Fucus vesiculosus*) $\delta^{15}N$ values trace decrease in sewage influence. Ecological Applications 14(2), 517-526.

Savage, C., 2005. Tracing the influence of sewage nitrogen in a coastal ecosystem using stable nitrogen isotopes. Ambio 34(2), 145-150.

Stevenson, R.J., Bothwell, M.L., Lowe, R.L., 1996. Algal ecology. Freshwater benthic ecosystems. Academic Press, San Diego, pp. 781.

Tewfik, A., Rasmunsen, J.B., McCann, K.S., 2005. Anthropogenic enrichment alters a marine benthic food web. Ecology 86(10), 2726-2736.

Viana, I.G., Fernández, J.A., Aboal, J.R., Carballeira, A., 2011. Measurement of $\delta^{15}N$ in macroalgae stored in an environmental specimen bank for regional scale monitoring of organic contamination in coastal areas. Ecological Indicators 11, 888-895.

Vizzini, S., Mazzola, A., 2004. Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Marine Pollution Bulletin 49, 61-70.

Voβ, M., Struck, U., 1997. Stable nitrogen and carbon isotopes as indicator of eutrophication of the Oder River (Baltic Sea). Marine Chemistry 59, 35-49.

Anexo III

Marine Environmental Research 72 (2011) 196–203

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Influence of salinity on fertilization and larval development toxicity tests with two species of sea urchin

C. Carballeira[a,*], L. Martín-Díaz[a,b], T.A. DelValls[a]

[a] UNITWIN/UNESCO/WiCoP. Physical Chemistry Department, University of Cádiz, 11510 Puerto Real, Cádiz, Spain
[b] Andalusian Center of Marine Science and Technology (CACYTMAR), Campus Universitario de Puerto Real, 11510 Puerto Real, Cádiz, Spain

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form
6 August 2011
Accepted 19 August 2011

Keywords:
Standardized tests
Paracentrotus lividus
Arbacia lixula
Confounding factor
Bioassay
Fertilization membrane

ABSTRACT

Sea urchin embryo-larval development (ELD) and fertilization tests have been widely used in ecotoxicity studies and are included in regulatory frameworks. Biological processes occur naturally within a range of salinity that depends on the species considered. In an attempt to determine the optimum range of salinity, ELD and fertilization bioassays were performed at different salinities (15–40.5‰) with two species of Atlantic sea urchin: *Arbacia lixula* and *Paracentrotus lividus*. In the ELD assay, the optimum range of salinity was wider for *A. lixula* (29–35.5‰) than for *P. lividus* (29–33‰). In the fertilization assay with *P. lividus* as a bioindicator species, the highest percentage of fertilization (90%) was obtained at salinities of between 29 and 33‰. More research on *A. lixula* is required, since the fertilization success was below 60%. The results of the present study demonstrate that salinity may be a confounding factor in interpreting ELD test results.

1. Introduction

Bioassays with marine organisms have been developed as a cost-effective method of evaluating marine water and sediment samples from contaminated sites. Standardized toxicity tests are useful in coastal ecosystem management, but require a readable endpoint to be established and identification of any confounding factors that may interfere with the measured response (OSPAR Comission, 2007). Bioassays enable detection of the effect of contaminants in the environment by measuring the responses of marine organisms, particularly at early life stages (His et al., 1999). Furthermore, bioassays are preferable to chemical analyses as they provide information about the bioavailability and toxicity of metals and mixtures of substances to organisms (Peters et al., 2002). Toxicity tests with early life stages of aquatic organisms have been proposed as a faster and more cost-effective method of testing chemicals and environmental samples than chemical analysis (Dinnel et al., 1987). Newly hatched larvae constitute particularly critical and sensitive stages, because at hatching the embryos lose their protective membrane and are fully exposed to potential toxins (Beiras et al., 2003).

Sea urchin toxicity tests, which use fertilization and larval development endpoints, are considered worldwide as useful tools for assessing toxicity in marine environments. These acute toxicity tests have been applied to determine the toxicity of sediments (elutriated sediments and porewater) (Beiras et al., 2003; Cesar et al., 2004; Geffard et al., 2001) and sea water (Beiras et al., 2001; Saco-Álvarez et al., 2010) under laboratory conditions, and have been included and standardized by several national environmental agencies (Environment Canada, 2011; USEPA, 2002).

It is well known that, for the purposes of risk assessment, a multispecies approach toward ecotoxicological testing is fundamental for accurate environmental management and ecological risk assessment procedures (Van Straalen, 2002). Thus, tests should be standardized, include exposure in the water phase (for marine assessment), be reasonably practicable, costs should be appropriate for the amount of information obtained, and organisms should be indigenous and sensitive to a broad spectrum of contaminants (Peters et al., 2002).

Paracentrotus lividus Lamarck 1816 (rock sea urchin) is a species commonly used in marine toxicity tests. One important characteristics of this species as bioindicator is its wide distribution, throughout the Mediterranean Sea and in the north-eastern Atlantic (Boudouresque and Verlaque, 2007). *Arbacia lixula* Linnaeus 1758 (black sea urchin) has been found to share habitat with *P. lividus* in several zones, both in Mediterranean and Atlantic coasts (Boudouresque and Verlaque, 2007; Martínez-Pita et al., 2010; Privitera et al., 2008; Tuya et al.,

Abbreviation: ELD, Embryo-Larval development.

* Corresponding author. Tel.: +34 956016423; fax: +34 956016040.
E-mail addresses: carlos.carballeira@uca.es (C. Carballeira), laura.martin@uca.es (L. Martín-Díaz), angel.valls@uca.es (T.A. DelValls).

0141-1136/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2011.08.008

2007). Therefore, it may also be a good candidate for toxicity testing. Despite these species can share habitat (shallow waters on rocky shores), their niches appeared not to be totally overlapped because of different substrate and feeding preferences (Boudouresque and Verlaque, 2007; Régis, 1979; Tuya et al., 2007). Régis (1979) suggested that structural particularities of both *P. lividus* and *A. lixula* could be adaptations to face physical stress, since the former usually occurs on horizontal or slightly inclined substrates, while the latter usually occurs on vertical substrates. In the Mediterranean Sea, *P. lividus* has been reported to feed mainly on erect algae (fixed or free macroalgae), seagrass and particulate matter, and it can even create a type of ecosystem known as *sea urchin barrens*, characterized by the absence of macroalgae and the presence of encrusting algae (Boudouresque and Verlaque, 2007). *A. lixula*, on the other hand, has been reported to graze encrusting coralline algae and possibly sessile invertebrates (Boudouresque and Verlaque, 2007; Tuya et al., 2007). Nevertheless, differences on nutrition habbits may depend on the available food resources (Boudouresque and Verlaque, 2007; Privitera et al., 2008).

Embryo-larval bioassays have proved to be very sensitive indicators of seawater contamination (Bay et al., 1983) because larvae represent critical stages of life (Connor, 1972).

The use of fertilization and embryo-larval development (ELD) toxicity bioassays to assess marine sediments and seawater pollution in a monitoring procedure in coastal areas requires analysis of the sensitivity of native sea urchin bioindicator species, together with standardization and optimization of the toxicity tests, which in turn requires identification of the different parameters that may alter the accuracy of the readable endpoint.

Fertilization and larval development tests with *P. lividus* have shown good consistency between tests results and pollutant contents (Beiras et al., 2003). On the other hand, other endpoints have been described, such as changes in pigmentation (Bay et al., 1983), growth (Beiras et al., 2001; Saco-Álvarez et al., 2010), dynamics of the first cleavage (Vaschenko et al., 1999), developmental arrest and larval malformations (Arslan and Parlak, 2007; Carballeira et al., 2010), as well as bioaccumulation of metals in larvae (Radenac et al., 2001).

In contrast, *A. lixula* has not been widely used for toxicity testing (Arslan and Parlak, 2007; Castagna et al., 1981; Máximo et al., 2008), although in some studies it has been used in conjunction with *P. lividus* (Cesar et al., 2002, 2004; 2009; Marín-Guirao et al., 2005a, 2005b). However, no comparative study has been made of the differences in sensitivity between the two species.

As regards the identification of physicochemical parameters that may alter the accuracy of the measured endpoint, it is important to consider the salinity, pH, dissolved oxygen and concentration of ammonia, as these have been described as possible confounding factors that may lead to alteration of false positive results (Saco-Álvarez et al., 2010).

In this work, particular attention has been paid to salinity. It has been demonstrated that pollutant uptake, accumulation and toxicity decrease at high salinities (Barbieri, 2010; Shukla et al., 2007; Verslycke et al., 2003), and that salinity can modify thermal tolerance (Alderdice and Forrester, 1971). Besides, salinity may affect survival (Fernandez et al., 2006), reproduction, development (Kashenko, 2007; Nissling et al., 2006) and growth (Boeuf and Payan, 2001) of marine and estuarine organisms, embryos and larvae showing less tolerance to salinity changes than adults. Nevertheless, the tolerance ranges appear to depend on the origin of the adults (His et al., 1999), e.g. the optimal salinity for mediterranean *P. lividus* larval development was settled, depending on temperature, at 33‰ (24 °C) (His et al., 1997) and between 34 and 35‰ (18–20 °C) (Bressan et al., 1995), while for atlantic *P. lividus* it was between 28 and 34‰ (Pétinay et al., 2009). For this reason, establishing optimal ranges of salinity for the species in different geographical locations can be important.

The developmental patterns and spatial distribution of many marine invertebrates are known to be influenced by variations in salinity (Roller and Stickle, 1993), which is possibly influenced by larval tolerance to salinity. The US Environmental Agency (USEPA, 2002) recommends that any discharges to the sea should not generate changes in salinity of more than 4‰, at a local level, with the purpose of avoiding damage to marine life.

The main aim of the present study was to optimize fertilization and larval development toxicity tests with the sea urchin species *P. lividus* and *A. lixula* from the south-west of the Iberian Peninsula by establishing the optimum range of salinity for toxicity testing for each species and endpoint. Previous studies have proposed the optimization and standardization of the sea urchin tests, however, they have been focused on the influence of the gamete suspension characteristics or the laboratory material (Lera et al., 2006), the selection of the endpoint (Saco-Álvarez et al., 2010), and the culture of parent sea urchins (Nelson et al., 2010; Pétinay et al., 2009). The tolerance of the species to different salinities (i.e. whether they were stenohaline or euryhaline) was considered in determining whether differences in species sensitivity are only due to differences in dermal uptake or are also due to internal physiological mechanisms of the early life stages.

Moreover, the suitability of carrying out fertilization and larval development tests at different salinities in risk assessment procedure will be discussed, taking into consideration the results obtained and the cost-effectiveness of the bioassays.

2. Material and methods

2.1. Sample processing

A stock solution of artificial seawater of 40.5‰ salinity was prepared according to Lorenzo et al. (2002). The use of artificial seawater for sea urchin larval development test has been recommended when natural seawater is not available (His et al., 1999; Saco-Álvarez et al., 2010), it has been taken into consideration for the sea urchin fertilization test by Environment Canada (2011) and USEPA (2002), and it has been widely validated in numerous research works, e.g. Beiras et al., 2003; Bellas et al., 2008; Bellas 2008; Lopes et al., 2010; Losso et al., 2007; Neiheisel and Young, 1992; Radenac et al., 2001. The use of artificial water guarantees that test water is clean and ensures that physicochemical conditions for sea urchins are optimal, so there will be no confounding factors when evaluating the influence of salinity on fertilization and larval development.

Samples of different salinity (15, 18.5, 22, 25.5, 29, 31.5, 33, 35.5 and 38.5‰) were obtained by adding appropriate amounts of bidistilled water to the stock solution. The range of salinity used was determined on the basis of previous studies of the *P. lividus* ELD bioassay (His et al., 1999; Saco-Álvarez et al., 2010), in which it was concluded that a reference salinity of 33‰ should be used. Incubation vials were filled with 20 mL or 10 mL of water of different salinity, in quadruplicate, for the ELD and fertilization bioassays respectively. Natural photoperiod, dissolved oxygen in excess (>95%) and temperature (20 °C) were the same for all samples and the pH did not vary by more than one unit (±8), within the range that allows normal development (Saco-Álvarez et al., 2010). Furthermore, the pH of the water was not significantly different between the start and the end of the experiment. Vials were kept in darkness at 4 °C until the bioassay was conducted.

2.2. Fertilization test

Adult sea urchins of both species were collected in the intertidal zone of a pristine area located at the outter part of Algeciras Bay, far from sources of contamination. Echinoderms were transported to

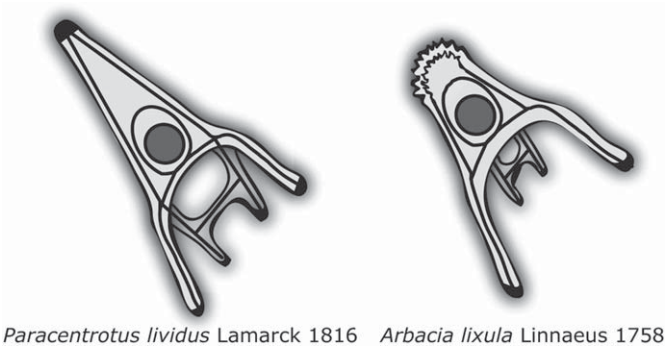


Fig. 1. Representation of normal larval shape of *Paracentrotus lividus* and *Arbacia lixula*, after 48 and 72 h development, respectively, at 20 °C.

the laboratory, in cold and dry conditions in order to avoid prior fecundation, and then were acclimated in aquaria with aerated clean seawater at 15 °C for 10 days.

Gametes were obtained from both species by injection of 1 mL KCl (0.5 M) through the peri-oral membrane of three specimens of each sex. Pools of eggs and sperm were prepared.

The fertilization procedure was adapted from that described by Volpi Ghirardini et al. (2001). Gamete maturity (spherical eggs and mobile sperm) and the density of both eggs and sperm was determined by observation under a microscope (OLYMPUS CKX41) at 40× and 4×, respectively, by using an hemocytometer when counting sperm. Subsequent gamete solutions were prepared according to two parameters: the desired egg concentration in the test solutions, which was 20 eggs/ml (200 eggs per vial) and the desired sperm:egg ratio, which was 20,000:1.

A volume of 100 µl of an adjusted suspension of 4×10^6 sperm was added to vials containing 10 ml of seawater solutions of different salinities (3 replicates each). After 60 min of exposure, approximately 200 eggs were transferred to the vials. Fertilization was allowed to take place for 20 min and was then blocked by adding one drop of 40% formaldehyde. Results were expressed as percentage of fertilized eggs (with fertilization membrane) after classifying one hundred eggs under a microscope.

2.3. Embryo development test

The same pools of gametes obtained during the fertilization test was used for the larval development test.

The *in vitro* fertilization methodology described by Fernández and Beiras (2001) was followed for both species. After most of the eggs (approx. 95%) were fertilized, volumes of the suspensions containing around 400 fertilized eggs were transferred into 20 ml incubation vials containing test solutions of different salinities (4 replicates each). Larval development was blocked by adding one drop of 40% formaldehyde at the moment that more than 90% of larvae in the control ASW reached the pluteus stage, after 48 h and 72 h incubation respectively for *P. lividus* and *A. lixula*.

Results were measured in each vial as the percentage embryological success. One hundred larvae were counted under a microscope, considering normal larvae as embryos with four arms fully developed and the same morphology as the reference embryos (i.e. with well-proportioned body parts) (Carballeira et al., 2010). The reference sample was established by prior observation of the samples of larger larvae with no abnormalities (Fig. 1). Abnormal larvae were also counted separately as undeveloped eggs or malformed larvae.

2.4. Statistical analysis

A one way ANOVA Tukey test (Zar, 1996) was applied to the data obtained in both bioassays to determine salinity-related differences in each species. A two-tailed Student *t* test for independent samples was used to determine the statistical significance of differences in resistance to salinity between species for each range of salinity (grouped according to salinity-related differences identified by the Tukey test) and toxicity test. The significance level was established at 95% ($p < 0.05$).

The application of Spearman non-parametric correlations enabled the influence of salinity (measured by determining the percentage of undeveloped and unfertilized eggs, and malformed larvae) and its significance to be determined for each toxicity test ($p < 0.01^{**}$ and $p < 0.05^{*}$). Statistical analyses were performed with SPSS software, version 17.0.

3. Results

The percentage of fertilized eggs and normal larvae determined at the range of salinities tested are shown for both bioassays performed with *P. Lividus* and *A. lixula*, in Fig. 2.

For both species and the range of salinity tested (15–40‰), fertilization occurred at above 18.5‰ and below 40.5‰, and larval development occurred at between 25.5 and 35.5‰.

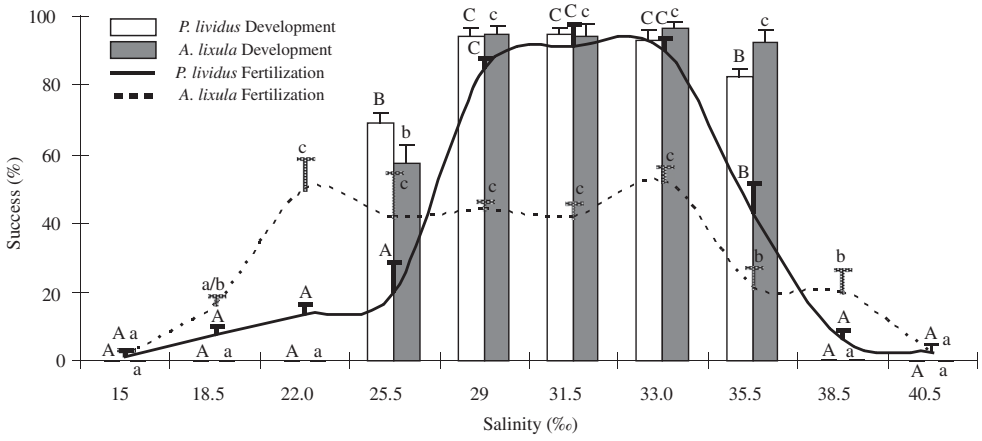


Fig. 2. Variation in the results of the embryonic development (Histogram) and fertilization bioassays (curves) with two species of sea urchin, *P. lividus* (white area and solid line) and *A. lixula* (black area and dashed line), in relation to salinity. The responses are grouped as a, b and c according to the statistical significance according to the Tukey test ($p < 0.05$), in capital letters for *P. lividus* and lowercase for *A. lixula*.

Table 1
Spearman correlation analysis. *, ** Significant at $p < 0.05$ and $p < 0.01$.

ELD (n = 40)	Malformed larvae				Undeveloped eggs			
	P. lividus		*A. lixula*		*P. lividus*		*A. lixula*	
	R^2	p	R^2	p	R^2	p	R^2	p
Salinity	0.004	0.973	0.117	0.3042	−0.221	0.062	−0.292*	0.016
Undeveloped eggs	−0.418**	0.000	−0.263*	0.03				

Fertilization (n = 30)	Unfertilized eggs			
	P. lividus		*A. lixula*	
	R^2	p	R^2	p
	−0.125	0.509	−0.085	0.657

R^2: Correlation values; p: Significance values; n: sampling size.

In the fertilization test with *P. lividus*, the highest percentage of fertilization (90%) occurred at salinities of 31 and 33‰. Fertilization was successful, although not optimal (values around 80%) at 29‰ but it did not differ significantly from fertilization success at 31–33‰. Fertilization of *A. lixula* eggs did not reach optimal levels and the maximum fertilization rate was achieved at salinities of22 and 33‰, with approximately 50% of fertilized eggs. Although fertilization of *A. lixula* eggs did not reach the values required for the development of the bioassay, this species was more tolerant to lower salinities (<25.4‰) than *P. lividus*.

The percentage of normal larval determined in *A. lixula* was optimal (≥90%) when organisms were exposed to salinities ranging from 29 to 35.5‰. Nevertheless, the range of salinity for optimum normal larva development (values ≥ 90%) was narrower in *P. lividus* (29–33‰).

When larval development occurred, significantly lower ($p < 0.05$) differences were observed for both species at salinities < 29‰. On the other hand, significant differences between species were observed for salinities >33‰. Thus, significantly lower values of larval development were observed at >33‰, for *P. lividus* and at >35.5‰ for *A. lixula*.

In both species there was a significant negative correlation between the number of undeveloped eggs and deformed larvae, while salinity only affected normally developed *A. lixula* eggs ($R = 0.292^*$) (Table 1). There was no significant correlation between salinity and the number of fertilized eggs ($R = -0.125$ and $R = -0.085$) in either species (Table 1), and the fertilization test was not therefore affected by salinity (Greenwood and Bennett, 1981).

According to the results of the Student t test (Table 2), both species can be used indistinctly in the ELD bioassay at salinities between 29 and 33‰. In the fertilization bioassay, significant differences were observed between species at almost all salinities tested (including at 25.5 and 35.5‰, at a significance level close to $P = 0.05$). Thus, results from the two species should not be compared for this bioassay.

4. Discussion

The sea urchin is an excellent biological indicator because of its remarkable sensitivity, its ease of availability and handling, synchronization of its development and the widespread knowledge of its embryology (Pavillon, 1988).

Most *in situ* and elutriation bioassays with sea urchins are developed under different physicochemical conditions in the sample sites and the reference site, considered as a clean location. Moreover, the quality of a toxicity test is defined by standards and requirements that ensure the well-being of the organisms during test development (Nascimento et al., 2002).

Factors associated with sediment storage (e.g. duration, temperature, container material, need for systematic and standardized storage) are known to be confounding factors (Nendza, 2002). Temperature, dissolved oxygen and recommended natural photoperiod (Fernández, 2002) were the same for all samples, and pH remained within the normal range for development (Saco-Álvarez et al., 2010). If the salinity of a sample is outside the optimum salinity range (e.g. brackish water), the results of the toxicity test will probably reflect unfavourable salinity rather than any toxic substance in the sample. Salinity is also an important variable that modifies the bioavailability of toxins (Sunda and Guillard, 1976) and their intrinsic toxicity (Riba et al., 2004). Increased metal toxicity in estuaries (exposed to periods of reduced pH and salinity) has been demonstrated by a sea urchin toxicity test (Fernández and Beiras, 2001).

4.1. Fertilization bioassay

Results from the fertilization test showed successful fertilization of *P. lividus* within a narrow range of salinity (the same range as for development of this species), whereas fertilization of *A. lixula* was always below 60%, possibly because of the difficulty in distinguishing the fertilization membrane (Fig. 3) (Máximo et al., 2008). The fertilization membrane in *P. lividus* is thicker and more easily observed than the membrane in the black sea urchin, which appears closely linked to the embryo, making it difficult to distinguish, and therefore altering the results of the bioassay. This appreciation agrees with the findings of Runnström et al. (1954), who observed that the fertilization membrane of *A. lixula* eggs was often closely adhered to the egg surface. The high adherence of the membrane does not impede the normal larval development but it difficults its identification.

Environment Canada (2011) described a method of developing a fertilization bioassay, with 5 different species of sea urchin (*Strongylocentrotus droebachiensis*, *Strongylocentrotus purpuratus*, *Dendraster excentricus*, *Arbacia punctulata* and *Lytechinus pictus*), in

Table 2
Differences between species for different salinities tested.

	Salinity (‰)									
Bioassay	15	18.5	22	25.5	29	31.5	33	35.5	38.5	40.5
ELD	1.000			0.007*	0.710			0.002*	1.000	
Fertilization	0.643	0.019*		0.069	0.000*			0.063	0.025*	0.678

Gray squares indicate significant differences between species, and white squares indicate no significant differences. *Significant differences at $p < 0.05$.

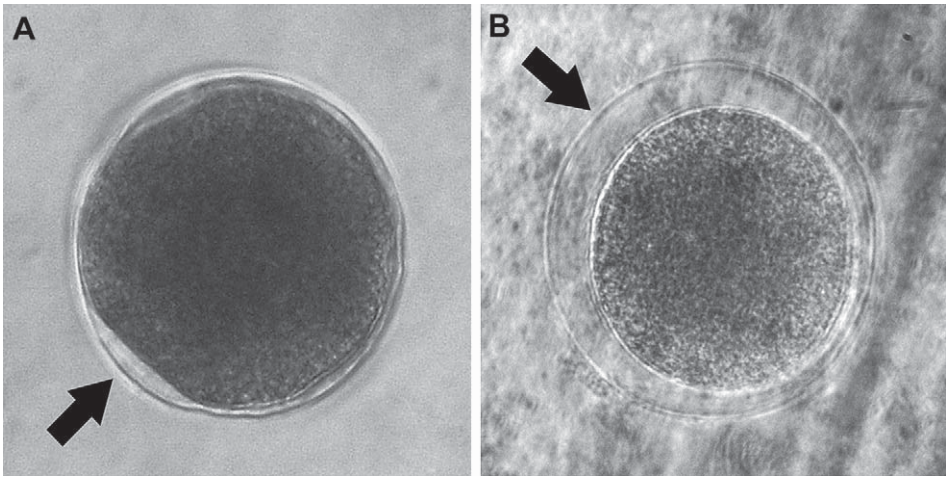


Fig. 3. Composite images of fertilized eggs of sea urchins: *Arbacia lixula* (left) and *Paracentrotus lividus* (right). The position of the fertilization membrane in each species is indicated by an arrow.

which it was recommended that the different species should develop at different temperatures (15 and 20 °C), salinity should be within the range 28–32 g/kg and difference from the reference salinity should not be higher than 1 g/kg. It was also recommended to carry out a standard test at a salinity of 30 g/kg. However, these requirements are not usually followed, e.g. when using elutriates or probably because the optimal conditions may differ between species and even between populations of the same species if they have different *geographical* origins (His et al., 1999).

Greenwood and Bennett, (1981) described some adverse effects of salinity on eggs of *Parechinus angulosus* (Leske), but found that sperm was scarcely affected by fluctuating salinity. In the present study, fertilization of *A. lixula* and *P. lividus* was not found to be affected by salinity (Spearman correlation analysis). Therefore the results will not reflect toxicity due to salinity, just toxicity due to pollutants, since the sperm is the only gamete exposed to the pollutants during the bioassay.

The physicochemical characteristics of an effluent will also have an impact on the development of the surrounding ecosystem since organisms require specific ecological niches, and therefore these characteristics should be taken into account when developing bioassays with regionally or locally occurring species.

Some pollutants do not affect formation of the fertilization membrane but have detrimental effects on larval development and may produce specific malformations (Carballeira et al., 2010). In general, the fertilization test has been described as less sensitive than the embryotoxicity test (Geffard et al., 2001; Heslinga, 1976; Losso et al., 2007; Xu et al., 2011) – as much as six times less sensitive for assessing metal toxicity (Xu et al., 2011). Increased acidification, different temperatures and pCO_2 did not reduce fertilization, and was only affected by sperm density (Byrne et al., 2010). The lowest pH at which significant effects on fertilization and cleavage were recorded was 7.6, while larval development was only affected at pH <7.4 (Moulin et al., 2011).

Because of the difficulties on distinguishing the fertilization membrane leading to misleading results, at least regarding *A. lixula*, and the lower sensitivity of the fertilization test in comparison with the larval development test, the fertilization test is not recommended, particularly if using *A. lixula*.

4.2. ELD bioassay

The recommended range of salinity for development of the *P. lividus* bioassay was previously established at 33–35‰ Mediterranean specimens (Volpi Ghirardini et al., 2001; Marín et al., 2001), and at 28–34‰ for atlantic specimens (Fernández, 2002; Pétinay et al., 2009; Saco-Álvarez et al., 2010). Several studies have addressed the effects of salinity on larval development of *P. lividus* (His et al., 1999; Saco-Álvarez et al., 2010), although only one study has considered how *A. lixula* is affected by salinity (Máximo et al., 2008). The optimum range of salinity (i.e. for development of more than 80% of four-armed larvae) reported for Mediterranean *P. lividus* was between 32 and 34‰ at 21 °C (His et al., 1999). In terms of growth (more than 80% of the maximum length of the larvae), the range of salinity for Atlantic specimens was established at between 30 and 40‰, a wider range than reported in previous studies conducted in this area, because abnormal larvae also develop and grow; the optimum salinity ranged between 31 and 35‰ according to maximum length values (Saco-Álvarez et al., 2010).

An optimum salinity range of 29–39‰ at 25 °C has been reported for *A. lixula* from the brazilian coast (Máximo et al., 2008). The present results, showing that *A. lixula* is less stenohaline (it tolerates salinities between 29 and 35.5‰) than *P. lividus* (which tolerates salinities between 29 and 33‰) are consistent with the previously reported ranges of salinity tolerance, regardless the origin of the tested specimens of *P. lividus*.

Differences in the recommended salinity for *P. lividus* and *A. lixula* may depend on the temperature, phenotypic characteristics of organisms and different seawater solutions. According to the results of the present study, there were no significant differences in sensitivity to salinity or tolerance for either species when ELD bioassay was carried out at between 29 and 33‰. Furthermore, there were no significant differences in the sensitivity to several chemical products used in land-based aquaculture between the two species (Carballeira et al., in press). Thus the results obtained with both species should be interchangeable and comparable, with preferential use of *A. lixula* because of its tolerance to a wider range of salinity. The recommended salinity and the duration of tests will depend on the regional temperatures where species live or experiments are performed. Nonetheless, the embryo development test is not suitable for assessing all types of salinity (e.g. brackish water).

4.3. Comparative applicability of the species considered

Differences between the sea urchin species considered, as regards selection of the most suitable for international ecotoxicity testing are listed in Table 3.

Table 3
Differences between sea urchins *Paracentrotus lividus* and *Arbacia lixula* and their use in the Embryo development and fertlization bioassays.

Bioassay characteristics	*Paracentrotus lividus*	*Arbacia lixula*
Fertilization bioassay	Fertilization rate higher than 90% at salinities between 29 and 33‰	Low Fertilization (below 60%). Diffuse membrane. Further research required
Time for larval development	48h at 20 °C	72h at 20 °C
Optimum salinity for ELD	Between 29 and 33‰ (stenohaline)	Between 29 and 35.5‰ (euryhaline)
Malformations identification	Easy	Onerous
Feeding	Mainly seagrass and erect algae (fixed or free)	Mainly encrusting coralline algae (fixed)
Geographical distribution	Atlantic coast, from England to NW Africa, and Mediterranean Sea.	Mediterranean Sea, Macaronesian Islands, Atlantic Coast (Western Africa and Eastern South America, Spain and Portugal).
Spawning	Once or twice a year (smaller quantityof gametes)	Continuous reproduction cycle (abundant gametes)

ELD: Embryo-larval development.

Differences in the grooves on the spines enable *P. lividus* to collect suspended particles on the spines and thrive in sites where food resources are scarce, whereas *A. lixula* meets its energy requirements by grazing on encrusting coralline algae and absorbing dissolved particles (Régis, 1979). Thus, *P. lividus* feeds on particles from throughout the environment (macroalgae, seagrass and small invertebrates) (Bulleri et al., 1999; Neill and Pastor, 1973). Bioassays carried out with *A. lixula* are likely to be more accurate, because the differences in feeding will better reflect the effects of pollution sources (incorporation of toxins through food), as observed in studies of accumulation in guts, gonads, and larvae, carried out by Quiniou et al. (1999), Radenac et al. (2001) and Russo et al. (2003). Food contaminated with Cd activates mechanisms for depurating metals in the echinoid coelom, so that more absorption of metals and other compounds takes place via seawater than via food in sea urchins (Warnau et al., 1995), and may even be greater than one order of magnitude higher in the case of PCB bioaccumulation (Danis et al., 2005). Bioaccumulation of pollutants has only been studied in *P. lividus* bioassays. Nonetheless, both species absorb pollutants through seawater, and should therefore be suitable for assessing sea water discharges.

The wider geographical distribution of *A. lixula*, which also coincides geographically with *P. lividus* (Boudouresque et al., 2001; Tommasi, 1966), makes this species more suitable for use in toxicity tests and for comparing results from different locations.

One major problem with this bioassay is the lack of availability of mature adults for obtaining sufficient numbers of gametes for the test. Guettaf et al. (2000) has described different spawning periods for *P. lividus* depending on the hydrodynamism in several areas, thus verifying two spawning periods: April–June and August–December. During the course of the present study, we observed that almost all black sea urchins injected with KCl spawned more gametes (both male and female) than the rock sea urchin, and that it was not necessary to open the specimens to collect the gametes. This reduced the waste of fresh biological material, wastage could be further reduced if gametes could be conserved for longer periods (Lera and Pellegrini, 2006). The differences in quantity of gametes can be explained by the lack of nutrients available for *P. lividus*, in which the relative size of the feeding apparatus is increased at the expense of gonad size (Fernandez and Boudouresque, 1997). Research carried out in Brazil with *A. lixula* found mature spawning male and female sea urchins throughout the entire year (continuous reproductive cycle) (Tavares, 2004; Zama and Ventura, 2005). Spawning of *P. lividus* mainly occurs once or twice a year depending on the location of organisms; the Atlantic Ocean and Mediterranean Sea respectively (Garmendia et al., 2010). Spawning of sea urchins also depends on the hydrodynamism in areas where they live (Guettaf et al., 2000). There is also some evidence of spawning throughout the whole year along the Algerian coast (Guettaf et al., 2000).

Studies with both species have shown the same sensitivity when exposed to samples with high levels of ammonium and reference toxicants such as cadmium, ammonium chloride, zinc sulfate and sodium dodecyl sulfate (Cesar et al., 2002). Other studies have shown similar sensitivity to certain toxins when different sea urchins species are compared (Xu et al., 2011).

The *P. lividus* development bioassay is shorter (48 h at 20 °C) than the *A. lixula* development bioassay (72 h at 20 °C), and abnormalities in the skeleton and shape appear to be more pronounced and are therefore easier to group in terms of the effect or pollutant (Carballeira et al., 2010). Normal larval shapes of each sea urchin are shown in Fig. 1, the characteristic shape of *P. lividus* makes it easier to identify different types of deformities.

The absence of significant differences between the sensitivity of the species considered (Carballeira et al., in press) enables comparison of results from both species at sites where one or another is not present. CETESB (1999) adapted the methodologies developed by different environmental agencies (Environment Canada, 2011; USEPA, 2002), because the species described are not present in Brazil. As *A. lixula* is present on both continents, its use as an indicator can be standardized. Further development of the use of locally occurring species should also be considered to improve the ecological significance of the test results, once intercalibrated and standardized.

5. Conclusions

Whole water and elutriated samples for toxicity bioassays must be of the same salinity, within the optimum range for each species, as this is required for the normal development of sea urchin embryos. *A. lixula* was found to be more tolerant to a wider range of salinity (29–35.5‰) than *P. lividus* (29–33‰).

Embryo development of *A. lixula* appears to be more suitable for ecotoxicity testing in terms of the optimum salinity range, geographical distribution, spawning and sensitivity, but further research is required to confirm this. More studies should be carried out with *A. lixula* fertilization test, in order to fine-tune the method.

Acknowledgements

This study was partly funded by the National Marine Aquaculture Plan. JACUMAR Project (2008): "Selection of Indicators, determination of reference values, design of programmes, protocols and Measures for environmental studies in aquaculture (INDAQUA)." Carlos Carballeira is grateful for funding from the University of Cadiz Predoctoral Fellowship Programme (Spain).

References

Alderdice, D., Forrester, C., 1971. Effects of salinity, temperature, and dissolved oxygen on early development of the Pacific Cod (*Gadus macrocephalus*). Journal of the Fisheries Research Board of Canada 28, 883–902.

Arslan, O., Parlak, H., 2007. Embryotoxic effects of nonylphenol and octylphenol in sea urchin *Arbacia lixula*. Ecotoxicology 16, 439–444.
Barbieri, E., 2010. Acute toxicity of ammonia in white shrimp (*Litopenaeus schmitti*) (Burkenroad, 1936, Crustacea) at different salinity levels. Elsevier, Amsterdam.
Bay, S.M., Oshida, P.S., Jenkins, K.D., 1983. A simple new bioassay based on echinochrome synthesis by larval sea urchin. Marine Environmental Research 8, 29–39.
Beiras, R., Vázquez, E., Bellas, J., Lorenzo, J.I., Fernández, N., Macho, G., Mariño, J.C., Casas, L., 2001. Sea-urchin embryo bioassay for *in situ* evaluation of the biological quality of coastal seawater. Estuarine, Coastal and Shelf Science 52, 29–32.
Beiras, R., Fernández, N., Bellas, J., Besada, V., González-Quijano, A., Nunes, T., 2003. Integrative assessment of marine pollution in Galician estuaries using sediment chemistry, mussel bioaccumulation, and embryo-larval toxicity bioassays. Chemosphere 52, 1209–1224.
Bellas, J., 2008. Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea-urchin embryo-larval bioassay. Aquatic Toxicology 88, 308–315.
Bellas, J., Fernández, N., Lorenzo, I., Beiras, R., 2008. Integrative assessment of coastal pollution in a Ría coastal system (Galicia, NW Spain): Correspondence between sediment chemistry and toxicity. Chemosphere 72, 826–835.
Boeuf, G., Payan, P., 2001. How should salinity influence fish growth? Comparative Biochemistry and Physiology Part C 130, 411–423. Toxicology & Pharmacology.
Boudouresque, C.F., Verlaque, M., 2007. Edible Sea Urchins: Biology and Ecology. In: John, M.L. (Ed.), Developments in Aquaculture and Fisheries Science. Elsevier, pp. 177–216.
Boudouresque, C.F., Verlaque, M., John, M.L., 2001. Ecology of *Paracentrotus lividus*Developments in Aquaculture and Fisheries Science. Elsevier. 177–216.
Bressan, M., Marin, M., Brunetti, R., 1995. Influence of temperature and salinity on embryonic development of *Paracentrotus lividus* (Lmk, 1816). Hydrobiologia 304, 175–184.
Bulleri, F., Benedetti-Cecchi, L., Cinelli, F., 1999. Grazing by the sea urchins *Arbacia lixula* L. and *Paracentrotus lividus* Lam. In the Northwest mediterranean. Journal of Experimental Marine Biology and Ecology 241, 81–95.
Byrne, M., Soars, N., Selvakumaraswamy, P., Dworjanyn, S.A., Davis, A.R., 2010. Sea urchin fertilization in a warm, acidified and high pCO_2 ocean across a range of sperm densities. Marine Environmental Research 69, 234–239.
Carballeira, C., Martín-Diaz, M.L., Del Valls, T.A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos con embriones de erizo, XII Foro dos recursos mariños e da acuicultura das rías galegas, O Grove.
Carballeira, C.B., De Orte, M., Viana, I.G., Del Valls, T.A., in press. Assessing the ecotoxicity of chemicals compounds associated to land-based marine fish farms: Sea urchin embryo bioassay with Paracentrotus lividus and Arbacia lixula. Ecotoxicity and Environmental safety.
Castagna, A., Sinatra, F., Scalia, M., Capodicasa, V., 1981. Observations of the effect of zinc on the gametes and various development phases of *Arbacia lixula*. Marine Biology 64, 285–289.
Cesar, A., Marín-Guirao, L., Vita, R., Marín, A., 2002. Sensitivity of Mediterranean amphipods and sea urchins to reference toxicants. Ciencias Marinas 28, 407–417.
Cesar, A., Marín, A., Marín-Guirao, L., Vita, I., 2004. Amphipod and sea urchin tests to assess the toxicity of Mediterranean sediments: the case of Portmán Bay. Scientia Marina 68, 205–213.
Cesar, A., Marín, A., Marin-Guirao, L., Vita, R., Lloret, J., Del Valls, T.A., 2009. Integrative ecotoxicological assessment of sediment in Portmán Bay (southeast Spain). Ecotoxicology and Environmental Safety 72, 1832–1841.
CETESB, 1999. Metodo de ensaio: Agua do mar-Teste de toxicidade cronica de curta duracao com Lytechinus variegatus, Lamark, 1816 (Echinodermata: Echinoidea). Cia. De Tecnologia de Saneamento Ambiental do Estado de Sao Paulo, São Paulo.
Connor, P.M., 1972. Acute toxicity of heavy metals to some marine larvae. Marine Pollution Bulletin 3, 190–192.
Danis, B., Cotret, O., Teyssié, J.L., Bustamante, P., Fowler, S.W., Warnau, M., 2005. Bioaccumulation of PCBs in the sea urchin *Paracentrotus lividus*: seawater and food exposures to a 14C-radiolabelled congener (PCB#153). Environmental Pollution 135, 11–16.
Dinnel, P.A., Link, J.M., Stober, Q.J., 1987. Improved methodology for a sea urchin sperm cell bioassay for marine waters. Archives of Environmental Contamination and Toxicology 16, 23–32.
Environment Canada, 2011. Biological Test Method: Fertilization Assay Using Echinoids (sea urchins and sand dollars), Method Development and Applications. Environmental Technology Center, Ottawa.
Fernández, N., 2002. Evaluación biológica de la contaminación marina costera mediante bioensayos con embriones del erizo de mar *Paracentrotus lividus*. Universidad de Vigo, Vigo, p. 211.
Fernández, N., Beiras, R., 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* Sea-Urchin. Ecotoxicology 10, 263–271.
Fernandez, C., Boudouresque, C.F., 1997. Phenotypic plasticity of *Paracentrotus lividus* (Echinodermata: Echinoidea) in a lagoonal environment. Marine Ecology Progress Series 152, 145–154.
Fernandez, C., Pasqualini, V., Boudouresque, C.-F., Johnson, M., Ferrat, L., Caltagirone, A., Mouillot, D., 2006. Effect of an exceptional rainfall event on the sea urchin (*Paracentrotus lividus*) stock and seagrass distribution in a Mediterranean coastal lagoon. Estuarine, Coastal and Shelf Science 68, 259–270.
Garmendia, J.M., Menchaca, I., Belzunce, M.J., Franco, J., Revilla, M., 2010. Seasonal variability in gonad development in the sea urchin (*Paracentrotus lividus*) on the Basque coast (Southeastern Bay of Biscay). Marine Pollution Bulletin 61, 259–266.
Geffard, O., Budzinski, H., Augagneur, S., Seaman, M.N.L., His, E., 2001. Assessment of sediment contamination by spermiotoxicity and embryotoxicity bioassays with sea urchins (*Paracentrotus lividus*) and oysters (*Crassostrea gigas*). Environmental Toxicology and Chemistry 20, 1605–1611.
Volpi Ghirardini, A.V., Novelli, A.A., Likar, B., Pojana, G., Ghetti, P.F., Marcomini, A., 2001. Sperm cell toxicity test using sea Urchin *Paracentrotus lividus* lamarck (Echinodermata: Echinoidea): sensitivity and discriminatory ability toward anionic and nonionic surfactants. Environmental Toxicology and Chemistry 20, 644–651.
Greenwood, P.J., Bennett, T., 1981. Some effects of temperature-salinity combinations on the early development of the sea urchin *Parechinus angulosus* (Leske). fertilization. Journal of Experimental Marine Biology and Ecology 51, 119–131.
Guettaf, M., San Martin, G.A., Francour, P., 2000. Interpopulation variability of the reproductive cycle of *Paracentrotus lividus* (Echinodermata: Echinoidea) in the south-western Mediterranean. Journal of the Marine Biological Association of the United Kingdom 80, 899–907.
Heslinga, G.A., 1976. Effects of copper on the coral-reef echinoid *Echinometra mathaei*. Marine Biology 35, 155–160.
His, E., Seaman, M.N.L., Beiras, R., 1997. A simplification the bivalve embryogenesis and larval development bioassay method for water quality assessment. Water Research 31, 351–355.
His, E., Heyvang, I., Geffard, O., de Montaudouin, X., 1999. A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassays for toxicological studies. Water Research 33, 1706–1718.
Kashenko, S., 2007. Adaptive responses of embryos and larvae of the heart-shaped sea urchin *Echinocardium cordatum* to temperature and salinity changes. Russian Journal of Marine Biology 33, 381–390.
Lera, S., Pellegrini, D., 2006. evaluation of the fertilization capability of *Paracentrotus lividus* sea urchin storaged gametes by the exposure to different aqueous matrices. Environmental Monitoring and Assessment 119, 1–13.
Lera, S., Macchia, S., Pellegrini, D., 2006. Standardizing the methodology of sperm cell test with *Paracentrotus lividus*. Environmental Monitoring and Assessment 122, 101–109.
Lopes, V., Fernández, N., Martins, R., Vasconcelos, V., 2010. Primary Screening of the Bioactivity of Brackishwater Cyanobacteria: Toxicity of Crude Extracts to *Artemia salina* Larvae and *Paracentrotus lividus* Embryos. Marine Drugs 8, 471–482.
Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquatic Toxicology 58, 27–41.
Losso, C., Novelli, A.A., Picone, M., Marchetto, D., Pantani, C., Ghetti, P.F., Ghirardini, A.V., 2007. Potential role of sulfide and ammonia as confounding factors in elutriate toxicity bioassays with early life stages of sea urchins and bivalves. Ecotoxicology and Environmental Safety 66, 252–257.
Máximo, M.V., Mottola, L.S.M., Resgalla- Jr., C., 2008. Sensibilidade do ouriço *Arbacia lixula* (Echinodermata: Echinoidea) em testes de toxicidade. Journal of the Brazilian Society of Ecotoxicology 3, 47–52.
Marín-Guirao, L., Atucha, A.M., Barba, J.L., López, E.M., Fernández, A.J.G., 2005a. Effects of mining wastes on a seagrass ecosystem: metal accumulation and bioavailability, seagrass dynamics and associated community structure. Marine Environmental Research 60, 317–337.
Marín-Guirao, L., Cesar, A., Marín, A., Lloret, J., Vita, R., 2005b. Establishing the ecological quality status of soft-bottom mining-impacted coastal water bodies in the scope of the water framework directive. Marine Pollution Bulletin 50, 374–387.
Marín, M.G., Da Ros, L., Moschino, V., Campesan, G., 2001. Sediment elutriate toxicity testing with embryos of sea urchin (*Paracentrotus lividus*). Aquatic Ecosystem Health & Management 4, 215–221.
Martínez-Pita, I., García, F., Pita, M.-L., 2010. Males and females gonad fatty acids of the sea urchins *Paracentrotus lividus* and *Arbacia lixula* (Echinodermata). Helgoland Marine Research 64, 135–142.
Moulin, L., Catarino, A.I., Claessens, T., Dubois, P., 2011. Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816). Marine Pollution Bulletin 62, 48–54.
Nascimento, I.A., Sousa, E.C.P.M., Nipper, M., 2002. Métodos em ecotoxicología marinha: Aplicaçoes no brasil. Ltda., A.G.e.I., Sao Paulo, p. 262.
Neiheisel, T.W., Young, M.E., 1992. Use of three artificial sea salts to maintain fertile sea urchins (*Arbacia punctulata*) and to conduct fertilization tests with copper and sodium dodecyl sulfate. Environmental Toxicology and Chemistry 11, 1179–1185.
Neill, F.X., Pastor, R., 1973. Relaciones troficas de *Paracentrotus lividus* (Lmk.) en la zona litoral. Investigación Pesquera 37, 1–7.
Nelson, R., Nipper, M., Lawrence, A., Watts, S., 2010. Parental dietary effect on embryological development response to toxicants with the sea urchin *Arbacia punctulata*. Bulletin of Environmental Contamination and Toxicology 84, 71–75.
Nendza, M., 2002. Inventory of marine biotest methods for the evaluation of dredged material and sediments. Chemosphere 48, 865–883.
Nissling, A., Johansson, U., Jacobsson, M., 2006. Effects of salinity and temperature conditions on the reproductive success of turbot (*Scophthalmus maximus*) in the Baltic Sea. Fisheries Research 80, 230–238.
OSPAR Comission, 2007. Background document on biological effects monitoring techniques, Assessment and Monitoring. OSPAR Commission, London, p. 122.
Pétinay, S., Chataigner, C., Basuyaux, O., 2009. Standardisation du développement larvaire de l'oursin, *Paracentrotus lividus*, pour l'évaluation de la qualité d'une eau de mer. Comptes Rendus Biologies 332, 1104–1114.
Pavillon, J.F., 1988. Influence de la toxicite du milieu d'elevage sur l'absorption d'alanine marquee au C14 par la larve pluteus de l'oursin *Paracentrotus lividus*. Oceanis 14, 391–397.

Peters, C., Becker, S., Noack, U., Pfitzner, S., Bülow, W., Barz, K., Ahlf, W., Berghahn, R., 2002. A marine bioassay test set to assess marine water and sediment quality—its need, the approach and first results. Ecotoxicology 11, 379–383.

Privitera, D., Chiantore, M., Mangialajo, L., Glavic, N., Kozul, W., Cattaneo-Vietti, R., 2008. Inter- and intra-specific competition between *Paracentrotus lividus* and *Arbacia lixula* in resource-limited barren areas. Journal of Sea Research 60, 184–192.

Quiniou, F., Guillou, M., Judas, A., 1999. Arrest and delay in embryonic development in sea urchin populations of the Bay of Brest (Brittany, France): link with environmental factors. Marine Pollution Bulletin 38, 401–406.

Régis, M.B., 1979. Particularités microstructurales du squelette de *Paracentrotus lividus* et *Arbacia lixula*: rapports avec l'écologie et l'éthologie de ces échinoïdes. Marine Biology 54, 373–382.

Radenac, G., Fichet, D., Miramand, P., 2001. Bioaccumulation and toxicity of four dissolved metals in *Paracentrotus lividus* sea-urchin embryo. Marine Environmental Research 51, 151–166.

Riba, I., DelValls Á., T., Forja, J.M., Gómez-Parra, A., 2004. The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam *Ruditapes philippinarum*. Environmental Toxicology and Chemistry 23, 1100–1107.

Roller, R.A., Stickle, W.B., 1993. Effects of temperature and salinity acclimation of adults on larval survival, physiology, and early development of *Lytechinus variegatus* (Echinodermata: Echinoidea). Marine Biology 116, 583–591.

Runnström, J., Wicklund, E., Löw, H., 1954. The fertilization and development of the sea urchin egg, *Arbacia lixula*, under the influence of fractions of egg homogenate. Experimental Cell Research 6, 459–478.

Russo, R., Bonaventura, R., Zito, F., Schroder, H.C., Muller, I., Muller, W.E., Matranga, V., 2003. Stress to cadmium monitored by metallothionein gene induction in *Paracentrotus lividus* embryos. Cell Stress & Chaperones 8, 232–241.

Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicology and Environmental Safety 73, 491–499.

Shukla, P., Gopalani, M., Ramteke, D., Wate, S., 2007. Influence of salinity on PAH uptake from water soluble fraction of crude oil in *Tilapia mossambica*. Bulletin of Environmental Contamination and Toxicology 79, 601–605.

Van Straalen, N.M., 2002. Threshold models for species sensitivity distributions applied to aquatic risk assessment for zinc. Environmental Toxicology and Pharmacology 11, 167–172.

Sunda, W.G., Guillard, R.R.L., 1976. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. Journal of Marine Research 34, 511–529.

Tavares, Y.A.G., 2004. Biologia reprodutiva dos equinóides Echinometra lucunter (Linnaeus, 1758) e Arbacia lixula (Linnaeus, 1758) na Ilha da Galheta, litoral paranaense, Brasil. Universidade Federal do Paraná, Curitiba.

Tommasi, L.R., 1966. Lista dos equinóides recentes do Brasil. Oceanografia Biológica 11, 1–50.

Tuya, F., Cisneros-Aguirre, J., Ortega-Borges, L., Haroun, R.J., 2007. Bathymetric segregation of sea urchins on reefs of the Canarian Archipelago: role of flow-induced forces. Estuarine. Coastal and Shelf Science 73, 481–488.

USEPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving water to west coast marine and estuarine organisms, third ed.. United States Environmental Protection Agency, Cincinnati, p. 370.

Vaschenko, M.A., Zhang, Z.P., Lam, P.K.S., Wu, R.S.S., 1999. Toxic effects of cadmium on fertilizing capability of spermatozoa, dynamics of the first cleavage and pluteus formation in the sea urchin *Anthocidaris crassispina* (Agassiz). Marine Pollution Bulletin 38, 1097–1104.

Verslycke, T., Vangheluwe, M., Heijerick, D., De Schamphelaere, K., Van Sprang, P., Janssen, C.R., 2003. The toxicity of metal mixtures to the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) under changing salinity. Aquatic Toxicology 64, 307–315.

Volpi Ghirardini, A.V., Novelli, A.A., Likar, B., Pojana, G., Ghetti, P.F., Marcomini, A., 2001. Sperm cell toxicity test using sea Urchin *Paracentrotus lividus* lamarck (Echinodermata: Echinoidea): Sensitivity and discriminatory ability toward anionic and nonionic surfactants. Environmental Toxicology and Chemistry 20, 644–651.

Warnau, M., Teyssie, J.L., Fowler, S.W., 1995. Effect of feeding on cadmium bioaccumulation in the echinoid *Paracentrotus lividus* (Echinodermata). Marine Ecology Progress Series 126, 305–309.

Xu, X., Li, Y., Wang, Y., Wang, Y., 2011. Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay. Toxicology In Vitro 25, 294–300.

Zama, P., Ventura, C.R.R., 2005. Comparação de caracteres morfológicos e reprodutivos do ouriço-do-mar Arbacia lixula (Echinodermata: Echinoidea) em duas localidades do litoral do Estado do Rio de Janeiro. VII Congreso de Ecología do Brasil, Caxambu, pp. 83–90.

Zar, J., 1996. Biostatistical analysis, 3 ed. Prentice Hall, Michigan.

Marine Environmental Research 77 (2012) 12–22

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents

C. Carballeira [a,*], J. Ramos-Gómez [b], L. Martín-Díaz [a,c], T.A. DelValls [a]

[a] UNITWIN/UNESCO/WiCoP, Physical Chemistry Department, CASEM, University of Cádiz, 11510, Puerto Real, Cádiz, Spain
[b] Ecotoxicology, Ecology Department, Faculty of Biology, University of Santiago de Compostela, 15782, Santiago de Compostela, A Coruña, Spain
[c] Andalusian Center of Marine Science and Technology (CACYTMAR), University of Cádiz, 11510, Puerto Real, Cádiz, Spain

ARTICLE INFO

Article history:
Received 4 November 2011
Received in revised form
31 December 2011
Accepted 5 January 2012

Keywords:
Paracentrotus lividus
Toxicity endpoint
Skeletal rods
Abnormal larva
Teratogenic effects
Aquaculture discharges

ABSTRACT

Standard toxicity screening tests are useful tools in the management of impacted coastal ecosystems. To our knowledge, this is the first time that the sea urchin embryo development test has been used to evaluate the potential impact of effluents from land-based aquaculture farms in coastal areas. The toxicity of effluents from 8 land-based turbot farms was determined by calculating the percentage of abnormal larvae, according to two criteria: (a) standard, considering as normal pyramid-shaped larvae with differentiated components, and (b) skeletal, a new criterion that considers detailed skeletal characteristics. The skeletal criterion appeared to be more sensitive and enabled calculation of effective concentrations EC_5, EC_{10}, EC_{20} and EC_{50}, unlike the classical criterion. Inclusion of the skeleton criterion in the sea urchin embryo development test may be useful for categorizing the relatively low toxicity of discharges from land-based marine fish farms. Further studies are encouraged to establish any causative relationships between pollutants and specific larval deformities.

1. Introduction

Most studies of the toxicity of effluents from land-based marine fish farms (LBMFFs) have focused on the analysis of nutrients, biological oxygen demand and suspended solids in water, although some have also considered chemicals and pathogens (Tello et al., 2010). The measurement of chemicals associated with land-based aquaculture is also very difficult owing to high dilution of the effluents. Inputs of chemicals to coastal ecosystems as a result of LBMFF activities may include prescribed compounds (pesticides and drugs), antifoulants, anaesthetics and disinfectants (Burridge et al., 2010). Nevertheless, the use of chemicals in aquaculture is not fully regulated and there are no official reports about which products are currently in use. Any study of the possible classes of contaminants present in the effluents would be extensive and time-consuming. Nonetheless, the information provided by chemical analysis is not sufficient to explain the potential effects of aquaculture on ecological processes in aquatic systems (Sarà, 2007). To assess the environmental impact of aquaculture discharges, standardized toxicity tests should be applied as these have been proven to be useful tools in the management of coastal ecosystems affected by human activities (Peters et al., 2002). In particular, standard screening bioassays offer several advantages over chemical analyses. As regards the significance of the information provided, bioassays reflect the bioavailability of contaminants (Hernando et al., 2007) as they integrate the effects of emerging pollutants not previously taken into account, the effects of chemical degradation products and the effects derived from the interactions between different pollutants (additive, synergistic and antagonistic effects) (Richardson et al., 2007; Wolska et al., 2007). From a functional point of view, bioassays are usually rapid (the biological response usually takes between a few hours and a few days), simple (organisms are easy to maintain in the laboratory prior to testing), the methodology is well defined (endpoints are easily measurable) and cost-effective (low requirements of skilled staff, space, material and the possibility of evaluating several samples at once).

The sea urchin embryo development test is a standard chronic toxicity bioassay advocated to be a cost-effective and useful method for use in screening the toxicity of specific pollutants, mixtures of these and natural matrices, and has regularly been used to evaluate the toxicity of water and sediments (Beiras et al., 2003; Cesar et al., 2009). This test consists of the study of teratogenic effects in early embryo to larval stages. Such effects appear to take place when stressor agents in the environment overcome the

* Corresponding author. Tel.: +34 956016423; fax: +34 956016040.
E-mail addresses: carlos.carballeira@uca.es (C. Carballeira), laura.martin@uca.es (L. Martín-Díaz), angel.valls@uca.es (T.A. DelValls).

0141-1136/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2012.01.001

protective mechanisms of sea urchin embryos and larvae. The standardized or classical criterion for evaluating toxicity by means of this test involves distinguishing between normal larvae, i.e., pyramid-shaped larvae with skeletal rods that are half the length of the long axis of the larvae, a differentiated gut and incipient post-oral arms, and deformed larvae, i.e., larvae that display blocked or delayed embryonic development, undifferentiated or abnormal gut and absent or abnormal skeleton (USEPA, 1994; Warnau et al., 1996). However, observation of only skeletal anomalies may be more rapid, sensitive and ecologically relevant than use of the classical criterion (without considering skeletal abnormalities), which, moreover may be affected by the determining role of food availability in the larval form, rate of growth of body parts and timing of development (Strathmann et al., 1992).

The sensitivity of skeletogenesis to specific contaminants has been widely demonstrated. Skeletal alterations (size reduction and malformations) have been observed after exposure to metals (Moureaux et al., 2011; Radenac et al., 2001; Warnau et al., 1996), linear alkylbenzene sulphonate (Bressan et al., 1991), pharmaceuticals (Graillet and Girard, 1994), biocides such as pesticides and antifoulants (Marín et al., 2007; Moschino and Marin, 2002; Pesando et al., 2003), and also UV radiation (Bonaventura et al., 2005). The sensitiveness and usefulness of sea urchin developmental malformations have been proven in monitoring different types of effluents and coastal waters (Guillou et al., 2000; Kobayashi and Okamura, 2004; Marín et al., 2007; Quiniou et al., 1999; Soualili et al., 2008; Trieff et al., 1995). Nonetheless, these morphological abnormalities have rarely been categorized as putative effects due to specific pollutants or their mixtures.

The ecological relevance of skeletal anomalies for evaluating environmental risk relies on several factors. Formation of the larval skeleton is a central event in sea urchin morphogenesis (Decker and Lennarz, 1988). The skeleton supports the larval body and determines its shape (Ettensohn and Malinda, 1993), which has been suggested to play a role in orientation and swimming (Pennington and Strathmann, 1990). Early skeletal alterations may decrease the ability of larvae to swim and respond to turbulence. Such alterations may also make feeding, predator avoidance and settlement more difficult than in normal larvae (O'Donnell et al., 2010). Thus, skeleton formation is essential for larval survival and consequently, the continuity of the sea urchin population. Sea urchin populations play a key role in shallow rocky shore communities. Variations in sea urchin density may severely alter the community structure of coastal ecosystems. Echinoids are a source of food for many species (Pearse, 2006), and have been found to determine algal assemblages on rocky shores (Hernández et al., 2008; McClanahan et al., 1996; Palacín et al., 1998) and to influence several invertebrate and fish populations (McClanahan et al., 1996) because of their grazing activity (Ebert, 1977) and because of inter-species competition (Pearse, 2006).

In the present study, the sea urchin embryo development bioassay was performed to evaluate the toxicity of effluents from eight turbot (*Psetta maxima* L.) LBMFFs located in Galicia (NW Spain). The sea urchin *Paracentrotus lividus* Lamarck (1816), which has already been validated for use in toxicity assessment (Beiras et al., 2003; Kobayashi and Okamura, 2004), was used in the study. In our experience, the shape of the larvae of *P. lividus* is one of the simplest and most stylized among the sea urchins that occur in Spain, which facilitates the identification of abnormalities.

The specific objective of this study was to determine the most useful and sensitive toxicity criteria to be applied in the sea urchin embryo development test. Two criteria were compared, the classic or standardized criterion (without considering skeletal deformities) and the improved or skeletal criterion (considering only skeletal defects). This objective was achieved by discerning which criterion best differentiated the effluents from each other and from the control treatment, as well as by determining the level of significance of the correlations between percentages of abnormal larvae and effluent concentrations. Selection of an appropriate criterion is especially important when assessing effluents of relatively low toxicity (as in the case of LBMFF effluents), because only highly sensitive criteria will enable discrimination of the different toxicities of effluents. The general objective was to test the suitability of the sea urchin embryo development bioassay, according to its sensitivity and cost-effectiveness, for inclusion in a monitoring plan, together with other bioassays (Carballeira et al., 2011a, 2011b), as a routine tool for the environmental surveillance of effluents from LBMFFs.

Although the sea urchin embryo development test has occasionally been used to evaluate the impact of cage-based fish farms (Marín et al., 2007), it has not been used to monitor the impact of LBMFFs.

2. Materials and methods

2.1. Effluent sampling

Effluent samples were collected from eight land-based turbot farms in Galicia (NW Spain) (Fig. 1). All farms have an open water circulation system, except for fish farm VIII, which recycles water. Sampling was performed during the annual period of maximum productivity (September–October) to reflect the maximum potential impact of land-based aquaculture activities. Effluent samples were collected from the LBMFFs by use of a peristaltic pump (Gilson M312) located at the output channel. The pump was programmed to collect a sample of about 6 L of effluent before it was diluted in the receiving water, during the hours when fish were metabolically most active (8 a.m.–8 p.m.). The water samples were

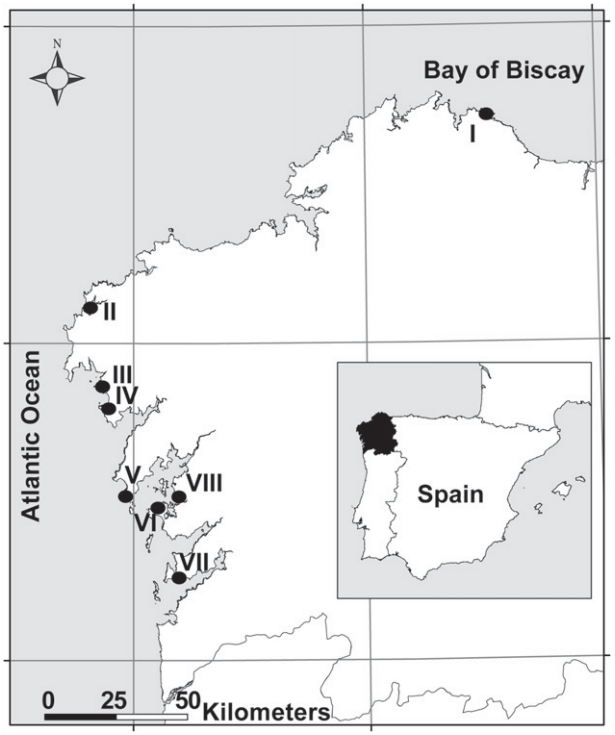


Fig. 1. Location of the land-based turbot farms from which the effluents under study were sampled.

then stored in 2 L pyrex bottles in darkness at 4 °C until the beginning of the test, within 48 h.

2.2. Effluent characterization

Physicochemical characterization of the LBMFF effluents involved analysis of salinity, pH, dissolved oxygen (DO), suspended solids (SS), total organic carbon (TOC), phosphates, nitrites, nitrates and ammonia. Salinity, pH and DO were determined by use of a multiparameter water quality meter (Hanna HI 9828). Analysis of SS, TOC, phosphates, nitrite and nitrate nitrogen and ammonia nitrogen and the quality controls were carried out according to EPA methods 1684, 415.1, 365.3, 353.2 and 350.1, respectively.

2.3. Sea urchin embryo development test

2.3.1. In vitro fertilization

Adult sea urchins of the species *P. lividus* were collected by scuba diving in a clean intertidal zone. In the laboratory, animals were transferred to pools continuously supplied with clean aerated sea water, for acclimation at 15 °C for 10 days. Gametes were obtained by injecting 1 mL of KCl (0.5 M) through the peri-oral membrane of three male and three female sea urchins. The fertilization procedure was as described by Fernández and Beiras (2001). Eggs from the three females were pooled and maintained in suspension with artificial seawater (Lorenzo et al., 2002), and sperm from the three males were pooled and maintained in a Petri dish in dry, cold conditions until fertilization. Prior to fertilization, gametes were examined under the microscope to check their maturity, i.e., that eggs were spherical and that sperm were highly mobile. The eggs were then transferred to a measuring cylinder containing artificial seawater and a few microlitres of dry pooled sperm were added. The mixture was carefully stirred to allow fertilization to take place. The density of fertilized eggs from control samples (artificial seawater) were counted under a microscope; fertilization success was approximately 95%.

2.3.2. Dilution preparation

Six different dilutions (volume of effluent: volume of artificial sea water) of each of the effluents from the fish farms were prepared in artificial sea water (Lorenzo et al., 2002): 0:1 (control), 1:20 (5%), 1:4 (25%), 1:2 (50%), 3:4 (75%) and 1:0 (100%). Solutions of ammonium chloride (NH_4Cl) and zinc sulphate ($ZnSO_4$) were also used as reference substances in the test, in accordance with published guidelines (Environment Canada, 1997; CETESB, 1999), to validate the accuracy of the tests. The solutions of these substances were prepared with the same artificial seawater. Nominal concentrations were 0.25, 0.5, 1, 2, 4, 8, 15 mg L^{-1} for ammonium chloride, and 0.01, 0.02, 0.05, 0.1, 0.5 mg L^{-1} for zinc sulphate.

2.3.3. Toxicity test

Vials (20 mL) were filled with the effluent dilutions and the reference substance solutions in quadruplicate. Approximately 400 fertilized eggs were placed in each vial. The vials were incubated for 48 h at 20 °C under natural photoperiod (Fernández and Beiras, 2001). Water quality parameters including temperature (20 ± 0.8 °C), salinity (34 ± 1‰), DO (>80%) and pH (7.6 ± 0.3) were measured at the beginning and at the end of the bioassay, to ensure the acceptability of the tests (Saco-Álvarez et al., 2010; Salamanca et al., 2009; Durán and Beiras, 2010).

After the incubation period, the larvae were fixed by adding one drop of 40% neutralized formalin, and were then observed in an inverted microscope (OLYMPUS CKX41). One hundred larvae were counted and developmental abnormalities recorded. Images were taken with a digital camera (OLYMPUS CAMEDIA C-5060).

2.4. Toxicity criteria

Developmental abnormalities were determined by applying two toxicity criteria:

i) Classical or standardized criterion: Larvae at pluteus stage, pyramid-shaped, with four fully developed arms (independently of the skeletal characteristics) and differentiated gut, were considered normal. Abnormal larvae were counted separately as undeveloped embryos (UD) or malformed larvae (MF). Undeveloped embryos included all the embryos that had not reach the pluteus stage after 48 h (fertilized eggs, blastula, gastrula and exogastrula), while malformed larvae included developed larvae with abnormalities.

ii) Skeletal criterion: Cone-shaped larvae at pluteus stage with four fully developed arms, with complete skeletal rods and with a skeleton of similar size to control larvae, were considered as normal larvae. Abnormal larvae were also counted separately within the aforementioned categories, undeveloped embryos and malformed larvae. Furthermore, in regard to specific abnormalities, this criterion distinguished different types of malformations: (a) crossed tip, (b) separated tip (c) fused arms, (d) incomplete or absent skeletal rods, (e) absence of skeletal rods and folded tip, (f) fractured ectoderm, (g) undeveloped embryos, i.e., fertilized eggs and blastula, gastrula, exogastrula and prepluteus stages (Fig. 2). Abnormalities were classified according to the severity of the alteration (Fig. 3). No toxicity (level 0 toxicity) was characterized by larvae without malformations. Slight toxicity (level 1 toxicity) was indicated by incorrect arrangement of skeletal rods of larvae. Toxicity was considered to be level 2 or moderate in larvae with no skeleton or in which skeletal rods were absent, incomplete or the shape was anomalous. Level 3 toxicity or high toxicity was characterized by blockage of development at early stages. Level 3 toxicity represents the most severe type of damage (Guillou et al., 2000; Warnau et al., 1996) since blastula and gastrula stages are retained by the absence of skeletal elements (Pennington and Strathmann, 1990).

Toxicity was quantified by counting the frequency of malformations detected. The toxicity was evaluated quantitatively by ranking the severity of deformations in larvae, as follows: 0 (none), 1 (slight), 2 (moderate) and 3 (high) (Carballeira et al., 2011c). A general index of toxicity (IT) was then calculated for each effluent, with the aim of summarizing the observed toxicity. The IT weights the degree of deformations by the frequency (%) observed in 4 replicates from each effluent, as follows: $IT = [0 \times \%\ \text{Level}\ 0 + 1 \times \%\ \text{Level}\ 1 + 2 \times \%\ \text{Level}\ 2 + 3 \times \%\ \text{Level}\ 3]/100$. The IT for each discharge ranged from 0 (no toxicity) to 3 (high toxicity).

2.5. Statistical analysis

The percentages of abnormal larvae (identified in each dilution of effluent and each reference substance solution by applying the classical and skeletal abnormality criteria) were analysed by the most suitable parametric model. Model suitability was evaluated according to four parameters: the maximum log likelihood value, Akaike's information criterion (AIC), the estimated residual variance and the *p*-value from a lack-of-fit test.

The effective concentrations (EC) EC_5, EC_{10}, EC_{20} and EC_{50} were calculated for the different effluents and the reference substances. The effective concentrations were defined as the concentrations of effluents or substances at which 5% (EC_5), 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}) of abnormal larvae are observed. These parameters

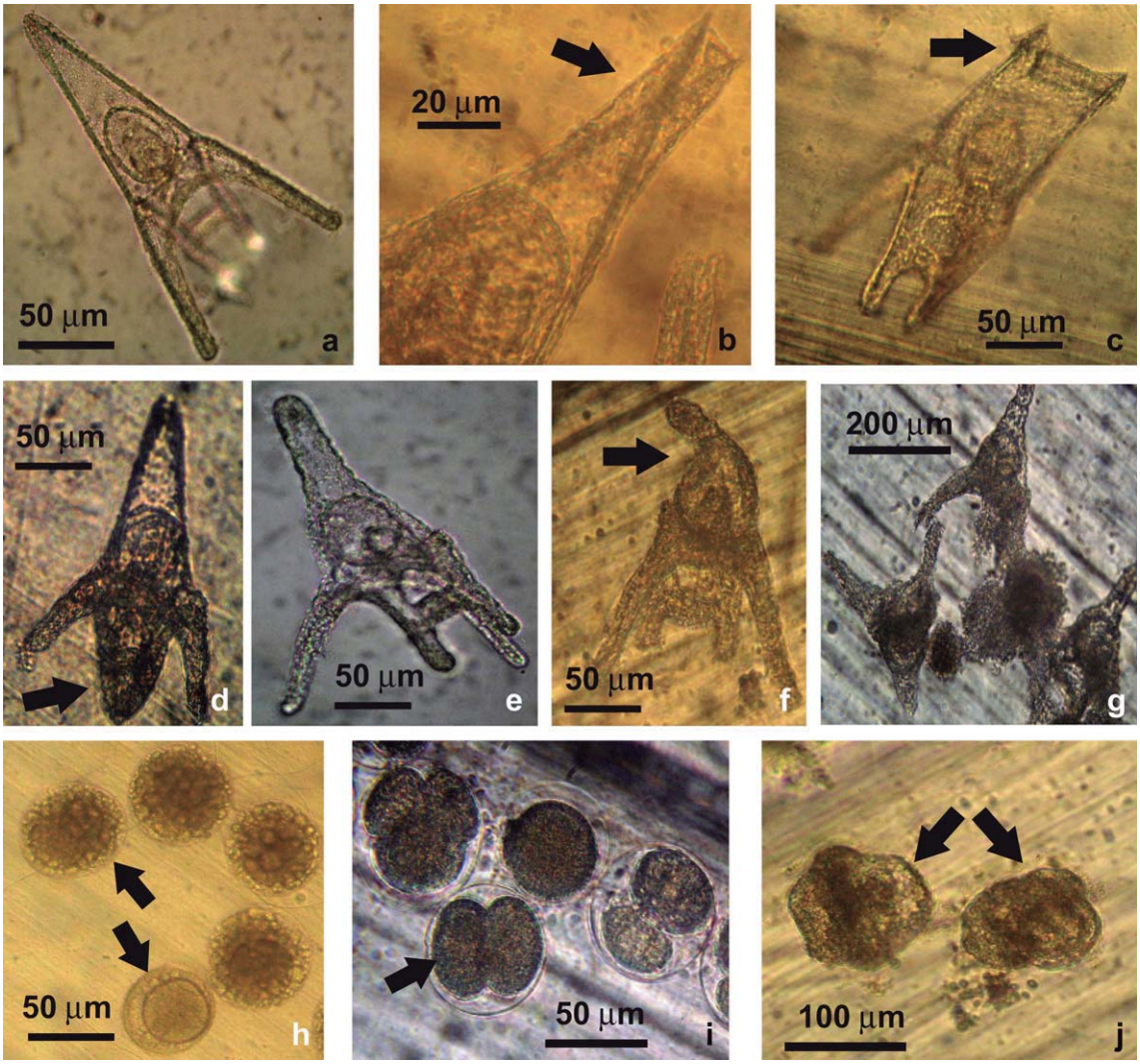


Fig. 2. Types of embryonic stages and developmental abnormalities of sea urchin *Paracentrotus lividus* observed in this study after 48 h incubation under controlled conditions of temperature, salinity, DO and pH. a: Normal larva at pluteus stage; b: Crossed tip; c: Separated tip; d: Fused arms; e: Incomplete or absent skeletal rods; f: Absence of skeletal rods and folded tip; g: Fractured ectoderm; h: Prepluteus; i, j: Undeveloped embryo.

were calculated by means of best fit dose–response curves obtained by non-linear regression analysis, by use of R software (R Development Core Team, 2008) and the dose–response curves (drc) add-on package (Ritz and Streibig, 2005).

Significant differences between the percentage of abnormal larvae in artificial seawater (control), in the dilutions of fish farm effluents and in the reference substance solutions were determined by one-way ANOVA followed by a Dunnett's test for multiple comparison. Three levels of significance were established: $p < 0.05$, $p < 0.01$ and $p < 0.001$. The effect of dilution on toxicity, according to both abnormality criteria (undeveloped embryos and malformed larvae, regardless of the type of malformation), was determined by a parametric Pearson correlation test. Significance was established at 95% ($p < 0.05$). Both ANOVA and Pearson correlation tests were performed with SPSS software (version 17.0).

3. Results

3.1. Effluent characterization

The physico-chemical characterization of the effluents discharged by the eight LBMFFs under study is shown in Table 1. Since single samples (24 h) do not represent the interannual variability on farms, most comparisons were of mean values of parameters obtained during a 6 year sampling period (data provided by the regional government of Galicia, Table 2).

As regards potentially confounding factors (salinity, pH, DO and phosphates) all effluents were generally within the optimal range for *P. lividus* embryo development (Böttger and McClintock, 2001; Saco-Álvarez et al., 2010). To our knowledge, the effects of nitrites and nitrates on embryo development have not previously been studied. However, the concentrations of these chemicals in the effluents were lower than the concentrations known to cause a decrease in adult sea urchin growth (Basuyaux and Mathieu, 1999) and in gonad development (Siikavuopio et al., 2004).

The discharge from fish farm VIII was the most concentrated, because of the intrinsic characteristics of this farm, and the parameters were therefore slightly different from the others. Nonetheless, with the exception of ammonia, the values of those parameters considered as confounding factors (salinity, pH, oxygen) were within the ranges that allow correct sea urchin larval development (Saco-Álvarez et al., 2010; Carballeira et al., 2011b). Slightly higher concentrations of phosphates and nitrates were found in effluents from farms II and VI respectively.

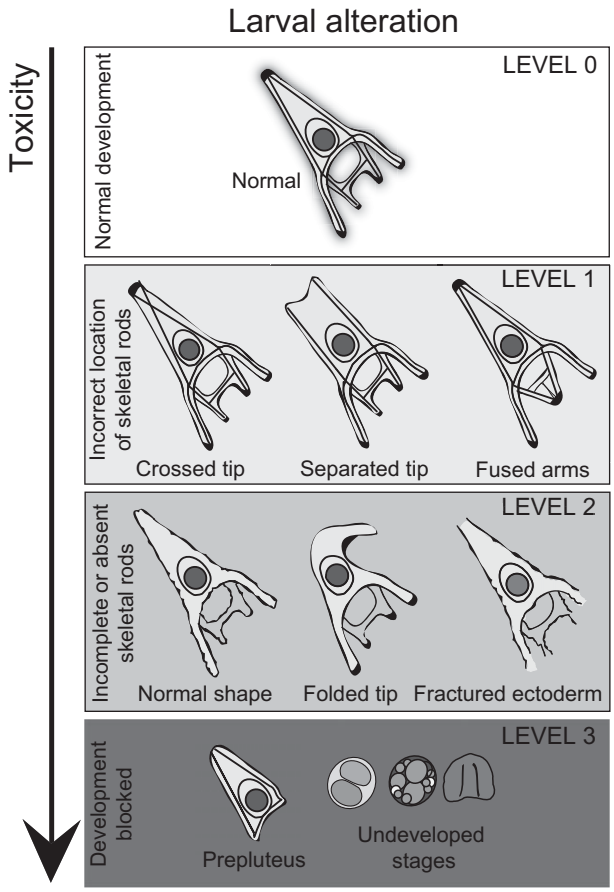


Fig. 3. Classification of larval malformations according to degree of alteration, in order to establish the severity of toxicity.

The ammonia content in all effluents was higher than that established as the LOEC = 0.08 mg L^{-1} (lowest observed effect concentration) (Saco-Álvarez et al., 2010).

3.2. Sea urchin bioassay

Controls contained at least 90% of normal larvae with both standard and skeletal criteria, indicating the validity of the test.

The best fit dose–response curves, which enabled calculation of the EC_5, EC_{10}, EC_{20} and EC_{50} for the reference substances, zinc sulphate and ammonium chloride, according to the classical and skeletal toxicity criteria, are shown in Fig. 4. The concentrated samples of reference substances blocked embryo development at early stages (undeveloped embryos, i.e., fertilized eggs and blastula, gastrula, exogastrula and prepluteus stages), and at the other concentrations, larvae did not display skeletal alterations, so the same ECs were obtained for each reference substance, regardless of the toxicity criterion used (Table 3). The EC values for both substances were consistent with those reported by other authors and by environmental agencies (Environment Canada, 1997; CETESB, 1999; Cesar et al., 2002). All percentages of abnormal larva corresponding to exposure to solutions were significantly different from control values.

The best fit dose–response curves, which enabled determination of the ECs for each farm effluent, according to the classical and skeletal toxicity criteria, are shown in Fig. 5. This figure also includes the significance of the larval abnormalities, relative to the control, using both toxicity criteria.

The results obtained with the classical toxicity criterion did not enable calculation of most EC values (Table 4) because, except for effluent I, the percentages of abnormal larvae did not exceed 50%, regardless of the effluent concentration. Reliable comparison of EC among effluents could not therefore be carried out. The highest dilution of effluents from fish farms I, II, IV and VIII yielded significantly higher percentages of malformed larva, than the control ($p < 0.05$). Significantly higher percentages of abnormal larva were obtained with 50% dilution of effluent III, 75% dilution of effluents V and VII, and undiluted effluent VI, than with the control ($p < 0.05$). The highest percentages of abnormal larva ($p < 0.05$) were obtained with the highest dilutions of effluent from Farm II.

Table 2
Averaged physico-chemical characteristics (period 2002–08) of the water entering the fish farm or input (I) and the effluent or output (O) from eighteen LBMFFs installed on Galician coast. Standard deviation (SD) and sampling size (n) are also indicated.

		Average	±SD	*n*
Salinity (g L^{-1})	I	34.3	0.4	179
	O	34.5	0.4	179
pH	I	7.98	0.48	179
	O	7.7	0.61	179
O_2 (mg L^{-1})	I	8.5	0.4	162
	O	8.3	0.2	162
O_2 (%)	I	85.9	7.1	162
	O	84.2	4.6	162
SS (mg L^{-1})	I	13	16	179
	O	18	12	179
TOC (mg L^{-1})	I	6.06	3.02	103
	O	7.63	1.94	103
Phosphates (mg L^{-1})	I	0.18	0.05	143
	O	0.24	0.04	143
Nitrites (mg L^{-1})	I	0.041	0.011	179
	O	0.075	0.016	179
Nitrates (mg L^{-1})	I	0.170	0.041	24
	O	0.198	0.022	24
Ammonia (mg L^{-1})	I	0.31	0.12	19
	O	0.56	0.42	19

Table 1
Physico-chemical characteristics of effluents from the LBMFFs I to VIII.

Fish farms	I	II	III	IV	V	VI	VII	VIII
Production (t yr^{-1})	2250	292	308	1194	348	44	285	189
Salinity (g L^{-1})	32.4	34.7	34.8	34.7	34.2	34.5	34.3	30.3
pH	7.48	7.92	7.94	7.63	7.84	7.64	7.59	7.71
DO (mg L^{-1})	8.3	8.42	8.51	7.83	8.92	8	8.23	7.93
DO (%)	79.6	80.1	82.2	79.1	87.3	79.5	80.3	78.4
SS (mg L^{-1})	23	17	18	21	16	18	14	39
TOC (mg L^{-1})	5.64	5.35	7.05	8.04	6.09	2.36	4.73	7.56
Phosphates (mg L^{-1})	0.22	0.423	0.251	0.292	0.241	0.211	0.231	0.473
Nitrites (mg L^{-1})	0.086	0.072	0.069	0.081	0.078	0.075	0.066	0.044
Nitrates (mg L^{-1})	0.191	0.200	0.192	0.205	0.141	0.536	0.206	0.287
Ammonia (mg L^{-1})	0.852	0.450	0.632	0.781	0.563	0.572	1.04	0.361

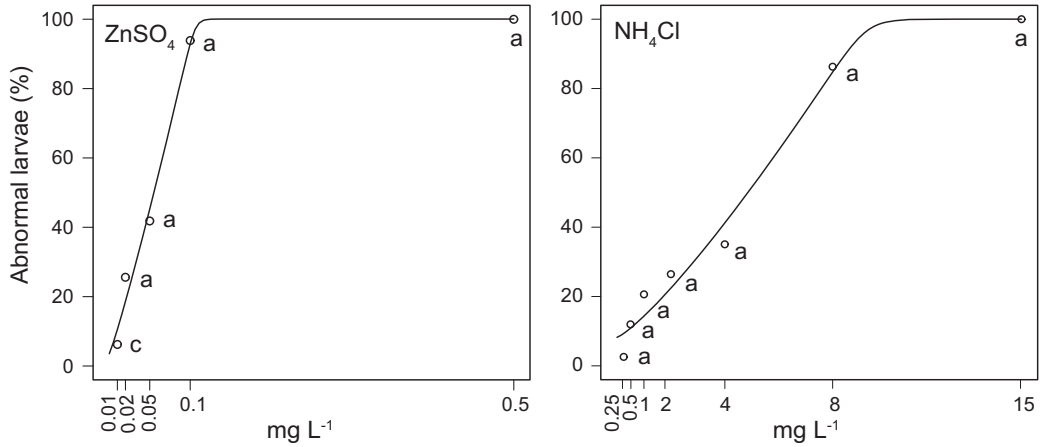


Fig. 4. Dose–response curves for reference substances NH_4Cl and $ZnSO_4$. Significant differences from controls are shown: a) $p < 0.001$***; b) $p < 0.01$**; c) $p < 0.05$*.

The percentages of malformed larvae determined by applying the skeletal criterion clearly increased with the effluent dilution (Fig. 5). Almost all the EC values were obtained for all the effluents (Table 4). The effluent ranking according to EC_5, EC_{10} and EC_{20} was I > VIII > V > II > VII > IV/III > VI. The ranking based on EC_{50} was slightly different, I > VIII > V > VII > III > II > IV > VI. The highest dilutions of effluents I and VIII yielded significantly higher percentages of abnormal larva than the control ($p < 0.05$). The 25% dilution of effluents II and V produced significantly higher percentages of malformed larvae than the control ($p < 0.05$). Significantly higher percentages of abnormalities were obtained with 50% dilution of effluents III, IV and VII, and 75% dilution of effluent VI than with the control ($p < 0.05$).

Correlations between effluent dilutions and specific malformations were determined: malformed larvae at the pluteus stage (according to classical and skeletal criteria), and undeveloped embryos (Table 5). The percentage of malformed larvae at the pluteus stage, determined by the skeletal criterion, reflected a greater influence of the effluent dilution than that determined by the classical criterion, again showing the lower sensitivity of the latter criterion. Skeletal malformations were significantly correlated ($p < 0.01$) with dilutions of effluents II, III, IV, V, VII and VIII, but not with effluents I and VI. All dilutions of effluent VI (except 75% dilution) were associated with a low percentage of abnormal larvae, while effluent I blocked early embryo development and the pluteus stage was not reached. Conversely, effluent dilution did not have any clear effect on the number of undeveloped embryos; fewer correlations with the dilutions were observed and the significance was lower, except for effluents I and IV.

The undiluted effluents caused specific skeletal malformations that varied from farm to farm. Characteristic skeletal malformations observed for each effluent are represented in the respective dose–response curves in Fig. 5. There were no developed larvae in the undiluted effluent from farm I, as embryo development appeared to be blocked at the gastrula stage (100%). Exposure to undiluted effluent from farm II led to more than 80% of larvae with the folded tip malformation. More than 90% of the abnormal larvae exposed to undiluted effluent from farms III and IV showed a fractured ectoderm. Cone-shaped larvae without skeletal rods were the most frequent type of abnormal larvae (>90%) produced by the undiluted effluent from farm V. The characteristic malformations (>80%) yielded by undiluted effluent from farms VII and VIII were crossed tip and separated tip, respectively. No specific skeletal malformation was associated with undiluted effluent from farm VI, although many of the larvae had fused arms (13%).

Further classification of the effluent toxicity was made according to the severity of the observed embryonic and larval alterations (IT) (Table 6).

Table 3
Effective concentrations (ECs) for reference substances. NH_4Cl and $ZnSO_4$ according to both toxicity criteria, classical and skeletal. Values are expressed as mg L^{-1} together with the model error.

	EC_5	EC_{10}	EC_{20}	EC_{50}
NH_4Cl	0.095 ± 0.063	0.470 ± 0.196	1.332 ± 0.351	4.270 ± 0.405
$ZnSO_4$	0.002 ± 0.0012	0.009 ± 0.003	0.021 ± 0.0045	0.055 ± 0.044

4. Discussion

Effluents from LBMFFs are complex mixtures of pollutants. They may contain pesticides (fungicides, algicides,…), disinfectants, antifoulants, antiparasitics, antibiotics, hormones, ammonia from fish urine, dissolved and suspended organic matter (fish feed and faeces) and biological contaminants (virus, bacteria and parasites). The chemical load of the selected LBMFFs has been demonstrated by Rey-Asensio et al. (2010). These authors showed that marine organisms in the surroundings of the LBMFFs bioaccumulated different classes of contaminants: the concentrations of metals Al, Cu, Hg and Ni were found to be significantly different in native *Anemonia sulcata* and transplanted *Saccharina saccharina* within a gradient of sampling sites affected by the LBMFF discharges, while oxolinic acid, amoxicillin, oxytetracycline and some pesticides (prometryn, prometon and chlorothalonil) were only bioconcentrated in *S. saccharina*.

However, analysis of the effluent composition is complex and of dubious effectiveness and efficiency: i) There are no official records in which LBMFF managers report the specific chemicals that they use (Burridge et al., 2010). An extensive screening analysis embracing a wide variety of contaminants would be required to determine the effluent composition. This would be very costly and not all chemicals could be measured; ii) Chemical products are used only occasionally, and so are not always present in the waste waters (Tello et al., 2010); and iii) Chemical concentrations in LBMFF effluents are usually low, and may even be below the detection limit (Rey-Asensio et al., 2010). Hence, chemical analysis may underestimate the toxic potential of effluents since some toxic substances may be present but not detected, others maybe toxic even at low concentrations, and synergic phenomena may also

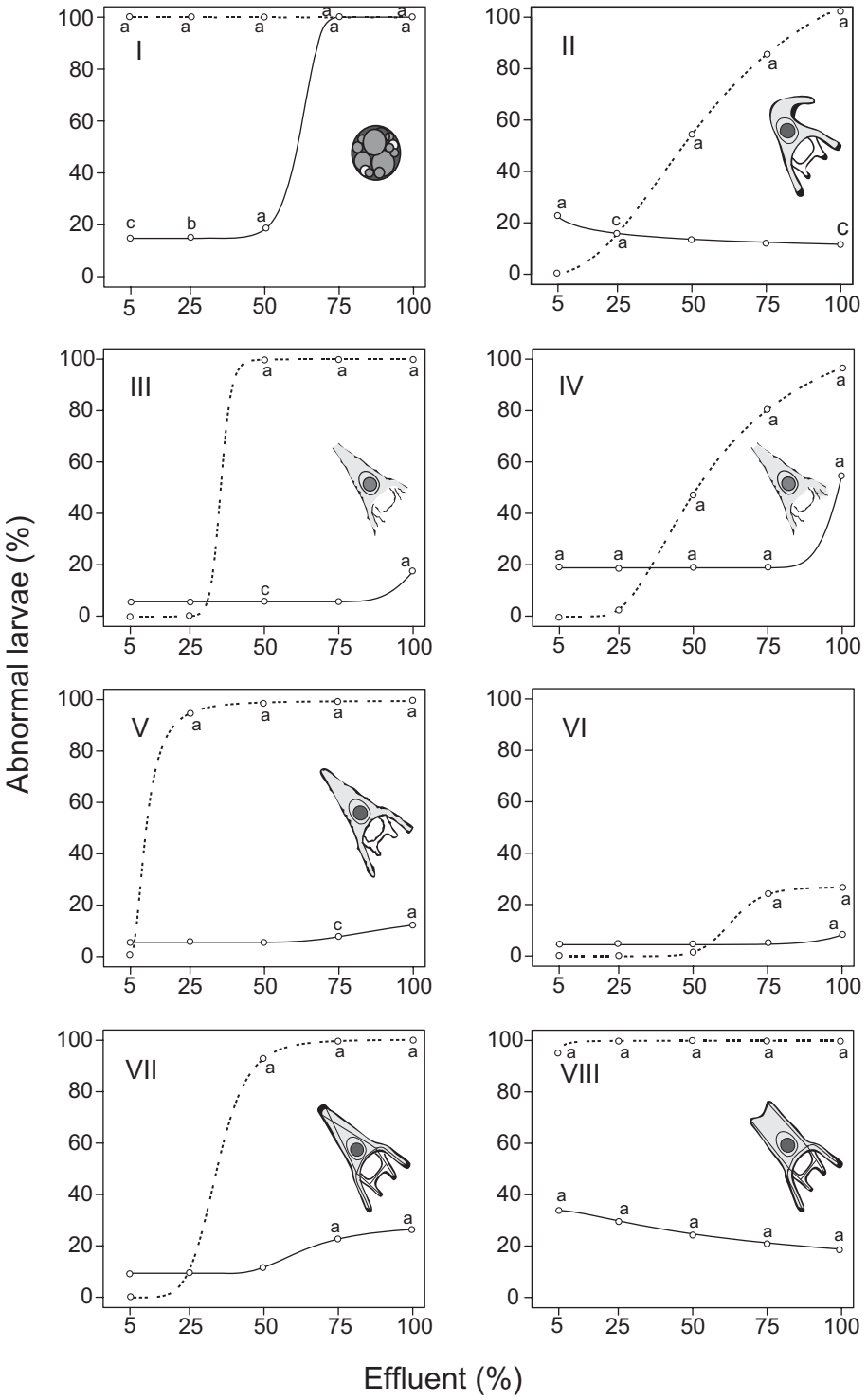


Fig. 5. Dose–response curves calculated for effluents I to VIII after applying classical (solid line) and skeletal (dashed line) criteria. The most frequent deformity observed in each effluent is represented in the corresponding graph: I: undeveloped embryos; II: larvae with absence of skeletal rods and folded tip; III: and IV: fractured ectoderm; V: incomplete or absent skeletal rods; VI: no abnormality prevailed; VII: crossed tip; VIII: separated tip. Significant differences when comparing with the control are shown: a) $p < 0.001$***; b) $p < 0.01$**; c) $p < 0.05$*.

occur. For these reasons, alternatives to chemical analysis for the assessment of LBMFF effluents are required.

In the present study, the potential impact of land-based aquaculture on the marine environment was assessed by evaluating the toxicity of the effluents from eight turbot land-based farms. The sea urchin embryo development bioassay was used for this purpose, for the first time. Two toxicity criteria were applied and compared in an attempt to contribute to the standardization of a toxicity criterion, sensitive to the particular mixtures of pollutants characterizing LBMFF effluents. Such standardization would enable detection of effluents of relatively low toxicity, thus avoiding the high variability in toxicity classification associated with the sea urchin

Table 4
Effective concentrations (ECs) obtained with the effluents from fish farms I to VIII according to classical and skeletal criteria. Values are expressed as effluent dilution percentage.

	Classical				Skeletal			
	EC_5	EC_{10}	EC_{20}	EC_{50}	EC_5	EC_{10}	EC_{20}	EC_{50}
I	–	–	50.9	55.9	<5	<5	<5	<5
II	–	–	–	–	14.1	19.4	27.2	46.4
III	–	–	–	–	30.6	31.7	32.9	35.3
IV	–	–	85.2	–	27.1	30.6	36.1	51.3
V	–	–	–	–	6.18	6.76	7.64	10.2
VI	55.2	–	–	–	55.6	60.2	68.7	–
VII	–	–	63.6	–	22	24.6	27.8	34.2
VIII	–	–	–	–	0.921	1.01	1.16	1.61

embryo development test, resulting from the different toxicity criteria used when counting deformities. Toxicity was characterized by use of two parameters: ECs and alterations in larval development.

EC values have been widely used to evaluate toxicity of different samples and types of chemicals and enable comparison between studies, although the present experimental data showed that the EC_x depended on the regression models and their accuracy decreased in the low effect zone (Sbrilli et al., 2005). Moreover, calculation of these endpoints requires preparation of serial dilutions and dose–response curves.

Identification of sea urchin malformations was carried out by applying the classical and the skeletal criteria. Both toxicity criteria identified the discharges from fish farms I and VI as the most and least toxic respectively. However, the results showed that the skeletal criterion was much more sensitive than the classical criterion for assessing the toxicity of fish farm effluents. The skeletal criterion indicated subtle alterations in the larval structure that enabled abnormalities to be correlated with effluent dilutions, and calculation of the effective concentrations.

There was great similarity between the classifications of toxicity according to skeletal abnormalities and EC values, which indicates that toxicity levels determined in this study were sufficiently sensitive and of practical use. Furthermore, by using the skeletal criterion, the toxicity of fish farm effluents could be determined more quickly than by calculation of ECs, because calculations and serial dilutions are not required. Moreover, the abnormalities themselves were found to indicate toxicity levels, depending on the

Table 5
Pearson correlation relating percentage of malformed larvae (MF) and percentage of undeveloped embryos (UD) with the effluent dilutions applying the two toxicity criteria, classical and skeletal. Significant differences at $^*p < 0.05$; $^{**}p < 0.01$.

		Classical criterion	Skeletal criterion
I	MF	**	–
	UD	*	**
II	MF	**	**
	UD	*	*
III	MF		**
	UD		
IV	MF	**	**
	UD	**	**
V	MF		**
	UD		
VI	MF	*	–
	UD	*	
VII	MF		**
	UD		
VIII	MF		**
	UD		

Grey spaces indicate inverse correlation with dilution.
– not determined.

Table 6
Toxicity of effluents from 8 turbot farms (I to VIII) according to the toxicity index calculated according the alteration level of larval structure.

Fish farm	Toxicity index	Toxicity
I	3.00	High
II	1.88	Moderate
III	2.10	Moderate
IV	1.98	Moderate
V	1.92	Moderate
VI	0.19	Absent
VII	0.86	Slight
VIII	1.04	Slight

severity of the alteration. This allowed classification of the effluent according to the potential toxicity, which could not be reliably achieved with the classical criterion.

The effects of pollutants and other stressors may be reflected in the sea urchin skeleton apparatus, either by the absence or the incorrect location of the skeletal rods, or by inactivation of the gene regulatory system underlying the development of the embryonic skeleton (Sharma and Ettensohn, 2010). Although many hypotheses have been presented (Garman et al., 1997; Graillet and Girard, 1994; Pesando et al., 2003; Pillai et al., 2003; Roepke et al., 2005), the current knowledge of sea urchin teratogenic mechanisms in response to specific chemicals is still very limited. Sea urchin skeletal rods are mineralized through amorphous calcium carbonate, finally forming a single crystal of calcite (Beniash et al., 1997). Some chemicals may react with carbonate and dissolve skeletal structures or affect mechanisms that control membrane permeability to calcium (Graillet and Girard, 1994).

Anomalies in sea urchin development have been observed to be overcome when exposure to the stressor agent ceases, in cases of UV radiation exposure (Amemiya et al., 1986). In contrast, some authors assume that this type of damage is irreversible (Nahon et al., 2009). We could not find any evidence of reversible effects in relation to chemical exposure, or whether injuries would disappear during metamorphosis. However, it appears clear that impairment of embryo-larval developmental reduces the ability of larvae to survive and will eventually affect the success of individual (O'Donnell et al., 2010; Pennington and Strathmann, 1990; Steevens et al., 1999) and population continuity.

Specific alterations of the skeleton may allow evaluation of the overall toxicity of effluents, but also the detection of specific contaminants or mixtures of these. However, there is little information available to help elucidate the possible composition of the effluents according to the observed malformations. Studies that characterize the teratogenic effects caused by specific contaminants are scarce. Furthermore, it is not known exactly what chemicals are used in aquaculture or the quantities applied (Burridge et al., 2010), and therefore researchers can only guess which products may be present in farm effluents. In further studies, deformities should be categorized according to the type of pollutant, in order to develop a rapid, cost-effective and representative screening test of the toxic characteristics of each farm.

Most physico-chemical parameters of all the effluents, with the exception of ammonia, were within the ranges considered not to alter the results of the test (Carballeira et al., 2011b; Saco-Álvarez et al., 2010), although the pH of effluent I (7.48) was close to the limit that may affect skeletogenesis (7.4) (Moulin et al., 2011). Although inorganic sodium phosphate and organic triethyl phosphate are known to cause abnormal development of sea urchins, individual specimens of the species *Lytechinus variegatus* survived at concentrations of 6 and 1000 mg L^{-1}, respectively (Böttger et al., 2006). The phosphate concentration in the effluents did not exceed 0.5 mg L^{-1}, so that this chemical was not likely to damage the

embryos in this bioassay. In severe experimental hypoxia, a large variety of skeletal malformations have been observed and often associated with functional anomalies (Burridge et al., 2010). However, dissolved oxygen was always higher than 78% in the fish farm effluents in the present study.

A high ammonia content was found in all the effluents because the values exceeded LOEC (0.08 mg L^{-1}) and EC_{10} values (0.91 mg L^{-1}) for this substance and species (Saco-Álvarez et al., 2010; Carballeira et al., unpublished results). Sea urchin embryos exposed to ammonia have been found to display crossed larval tip as a specific malformation (Carballeira et al., unpublished results). Ammonia may therefore be a major constituent of the effluent from farm VII.

Physico-chemical characteristics of effluents are not the only causes of deformities. Experiments with sea urchin embryos exposed to X-ray and UV demonstrated that radiation may affect skeletogenesis, e.g., abnormalities in skeletal rod elongation and patterning (Bonaventura et al., 2005; Moulin et al., 2011). However, any influence of radiation was ruled out in the present study because all incubation vials, including those containing the control samples, were exposed to a natural photoperiod under the same conditions. If any harmful radiation had been present, the control larvae would also have been malformed.

Ozone has been reported to cause skeletal malformations in organisms (Stalter et al., 2010). Farm VIII has a water recycling system and water flows through a closed circuit. Water is treated by ozonification before being recycled. The higher concentration of contaminants due to water recycling together with the use of ozone may explain the effluent characteristics and the high toxicity.

Graillet and Girard (1994) and Pesando et al. (2003) reported similar skeletal abnormalities in *P. lividus* to those observed in the present study after exposure of embryos to the herbicide 2,4,5-trichlorophenoxyacetic acid and to several neurotoxic insecticides. Diazinon was particularly closely associated with ectoderm fracturation and incomplete skeletal rods. In aquaculture, pesticides are used to prevent, destroy or repel any pest that may reduce fish production by spreading disease or competing for resources.

The neurotransmitter and calmodulin antagonist chlorpromazine, which is used as a sedative, was found to cause absence of skeletal rods and the formation of spicules in abnormal positions in larvae of *Lytechinus pictus* (Anitole et al., 1988). Although there is no evidence that this particular product is used in aquaculture, fish are often sedated during transport to decrease oxygen consumption and CO_2 and NH_3 production.

In addition to the studies cited, many others have investigated the effects of contamination on embryonic development in different species of sea urchin, but did not relate specific types of damage to specific contaminants. Antibiotics that are commonly used in aquaculture, such as tetracyclines and sulfonamides, have vbeen cited as a cause of human skeletal malformations (Tuchmann-Duplessis, 1984). However, only high concentrations of antibiotics appeared to have significant effects on skeleton formation in the sea urchin (Carballeira et al., unpublished results), and were not associated with specific skeletal damage. The most important metals in turbot aquaculture are Cd, Zn and Cu, as these are trace constituents of the fish diet, disinfectants, and antifoulants (Dean et al., 2007). Skeletal malformations of sea urchin larvae are known to be caused by zinc (Timourian, 1968) and cadmium chloride (Kobayashi and Okamura, 2004; Roccheri et al., 2004), and ternary and quaternary mixtures of these metals have additive effects (Xu et al., 2011), although specific malformations have not been reported. The gametes and embryos of the sea urchin species *P. lividus* and Psammechinus microtuberculatus have been used to study the teratogenic effects of chloramphenicol, nicotine, chlorpromazine, imipramine and thalidomide (Hagström and Lönning, 1973), although again, chemical exposure was not related to sea urchin malformations. A larval development bioassay has been carried out with *P. lividus* to evaluate the toxicity of sediments from *Sparus aurata* and *Tunna tunna* cage farms (Marín et al., 2007). Abnormalities of the skeleton were taken into account, but were not related to the type of contamination.

The sea urchin development test using the skeletal criteria has been found to be a sensitive and rapid method of assessing the toxicity of LBMFF effluents. Nevertheless, other studies that have evaluated the effluents from these particular LBMFFs have shown that toxicity classification may vary depending on the bioassay performed. Although the sea urchin bioassay with different species has been found to be sufficiently sensitive for use with fish farm discharges (Carballeira et al., 2011a), other bioassays should be conducted with several species from different trophic levels to monitor all pollutants from waste waters (Peters et al., 2002).

5. Conclusions

The effluents from LBMFFs are complex mixtures of metabolic wastes and contaminants of different nature. The sea urchin embryo development test is a rapid cost-effective method that enables assessment of the toxicity associated with LBMFF effluents. This bioassay, used with the skeletal criterion, is an excellent candidate for inclusion in a battery of bioassays designed to monitor discharges from LBMFFs because of its greater sensitivity and ecological relevance.

The identification of specific alterations during sea urchin embryonic development appears to be a promising method for the detection of contaminants or chemical mixtures, as well as for environmental monitoring of nearshore areas. Nevertheless, further research is required in order to establish the relationships between specific malformations and the pollutants or mixture of pollutants that cause them.

Acknowledgements

This study was partly funded by the National Marine Aquaculture Plan through the JACUMAR Project (2008), Selection of Indicators, determination of reference values, design of programmes, protocols and Measures for environmental studies in aquaculture (INDAQUA). C. Carballeira is grateful for funding from the University of Cadiz Predoctoral Fellowship Programme (Spain).

References

Amemiya, S., Yonemura, S., Kinoshita, S., Shiroya, T., 1986. Biphasic stage sensitivity to UV suppression of gastrulation in sea urchin embryos. Cell Differentiation 18, 45–49.

Anitole, K.G., Stahle, P.L., Ridenour, C.S., Lappas, N.T., Brown, K.M., 1988. Chlorpromazine-sensitive developmental processes in the sea urchin, *Lytechinus pictus*-I. Inhibition of cleavage, gastrulation and primary mesenchyme cell differentiation. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 90, 47–53.

Basuyaux, O., Mathieu, M., 1999. Inorganic nitrogen and its effect on growth of the abalone *Haliotis tuberculata* Linnaeus and the sea urchin *Paracentrotus lividus* Lamarck. Aquaculture 174, 95–107.

Beiras, R., Fernández, N., Bellas, J., Besada, V., González-Quijano, A., Nunes, T., 2003. Integrative assessment of marine pollution in Galician estuaries using sediment chemistry, mussel bioaccumulation, and embryo-larval toxicity bioassays. Chemosphere 52, 1209–1224.

Beniash, E., Aizenberg, J., Addadi, L., Weiner, S., 1997. Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. Proceedings of the Royal Society of London. Series B: Biological Sciences 264, 461–465.

Bonaventura, R., Poma, V., Costa, C., Matranga, V., 2005. UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochemical and Biophysical Research Communications 328, 150–157.

Böttger, S.A., Devin, M.G., Walker, C.W., 2006. Suspension of annual gametogenesis in North American green sea urchins (*Strongylocentrotus droebachiensis*)

experiencing invariant photoperiod-Applications for land-based aquaculture. Aquaculture 261, 1422–1431.
Böttger, S.A., McClintock, J.B., 2001. The effects of organic and inorganic phosphates on fertilization and early development in the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 129, 307–315.
Bressan, M., Marin, M.G., Brunetti, R., 1991. Effects of linear alkylbenzene sulphonate (LAS) on skeletal development of sea urchin embryos (*Paracentrotus lividus* Lmk). Water Research 25, 613–616.
Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K., 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306, 7–23.
Carballeira, C., De Orte, M., Viana, I.G., DelValls, T.A., unpublished results. Assessing the ecotoxicity of chemicals compounds associated to land-based marine fish farms: Sea urchin embryo bioassay with *Paracentrotus lividus* and *Arbacia lixula*. Archives of Environmental Contamination and Toxicology.
Carballeira, C., De Orte, M.R., VianaI, I.G., Carballeira, A., 2011a. Implementation of a minimal biological test set for assessment of ecotoxic effect of effluents from land-based fish farms. Ecotoxicology and Environmental Safety.
Carballeira, C., Martín-Díaz, L., DelValls, T.A., 2011b. Influence of salinity on fertilization and larval development toxicity tests with two species of sea urchin. Marine Environmental Research 72, 196–203.
Carballeira, C., Espinosa, J., Carballeira, A., 2011c. Linking $\delta^{15}N$ and histopathological effects in molluscs exposed in situ to effluents from land-based marine fish farms. Marine Pollution Bulletin 62, 2633–2641.
Cesar, A., Marín-Guirao, L., Vita, R., Marín, A., 2002. Sensitivity of Mediterranean amphipods and sea urchins to reference toxicants. Ciencias Marinas 28, 407–417.
Cesar, A., Marín, A., Marin-Guirao, L., Vita, R., Lloret, J., DelValls, T.A., 2009. Integrative ecotoxicological assessment of sediment in Portmán Bay (southeast Spain). Ecotoxicology and Environmental Safety 72, 1832–1841.
CETESB, 1999. Metodo de Ensaio: Agua do Mar-teste de Toxicidade Cronica de Curta Duracao com *Lytechinus variegatus*, Lamark, 1816 (Echinodermata: Echinoidea). Cia. De Tecnologia de Saneamento Ambiental do Estado de Sao Paulo, São Paulo.
Dean, R.J., Shimmield, T.M., Black, K.D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: An extensive survey. Environmental Pollution 145, 84–95.
Decker, G.L., Lennarz, W.J., 1988. Skeletogenesis in the sea urchin embryo. Development (Cambridge, England) 103, 231–247.
Durán, I., Beiras, R., 2010. Assessment criteria for using the sea-urchin embryo test with sediment elutriates as a tool to classify the ecotoxicological status of marine water bodies. Environmental Toxicology and Chemistry 29, 1192–1198.
Ebert, T.A., 1977. An experimental analysis of sea urchin dynamics and community interactions on a rock jetty. Journal of Experimental Marine Biology and Ecology 27, 1–22.
Environment Canada, 1997. Biological Test Method: Fertilization Assay using Echinoids (sea urchins and sand dollars). Method Development and Applications. Environmental Technology Center, Ottawa.
Ettensohn, C.A., Malinda, K.M., 1993. Size regulation and morphogenesis: a cellular analysis of skeletogenesis in the sea urchin embryo. Development 119, 155–167.
Fernández, N., Beiras, R., 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea urchin. Ecotoxicology 10, 263–271.
Garman, G.D., Anderson, S.L., Cherr, G.N., 1997. Developmental abnormalities and DNA-protein crosslinks in sea urchin embryos exposed to three metals. Aquatic Toxicology 39, 247–265.
Graillet, C., Girard, J.P., 1994. Embryotoxic potency of 2,4,5-trichlorophenoxyacetic acid on sea urchin eggs: Association with calcium homoeostasis. Toxicology In Vitro 8, 1097–1105.
Guillou, M., Quiniou, F., Huart, B., Pagano, G., 2000. Comparison of embryonic development and metal contamination in several populations of the sea urchin *Sphaerechinus granularis* (Lamarck) exposed to anthropogenic pollution. Archives of Environmental Contamination and Toxicology 39, 337–344.
Hagström, B.E., Lönning, S., 1973. The sea urchin egg as a testing object in toxicology. Acta Pharmacologica et Toxicologica 32, 1–49.
Hernández, J.C., Clemente, S., Sangil, C., Brito, A., 2008. The key role of the sea urchin Diadema aff. antillarum in controlling macroalgae assemblages throughout the Canary Islands (eastern subtropical Atlantic): an spatio-temporal approach. Marine Environmental Research 66, 259–270.
Hernando, M.D., De Vettori, S., Martínez Bueno, M.J., Fernández-Alba, A.R., 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere 68, 724–730.
Kobayashi, N., Okamura, H., 2004. Effects of heavy metals on sea urchin embryo development. 1. Tracing the cause by the effects. Chemosphere 55, 1403–1412.
Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquatic Toxicology 58, 27–41.
Marín, A., Montoya, S., Vita, R., Marín-Guirao, L., Lloret, J., Aguado, F., 2007. Utility of sea urchin embryo-larval bioassays for assessing the environmental impact of marine fishcage farming. Aquaculture 271, 286–297.
McClanahan, T.R., Kamukuru, A.T., Muthiga, N.A., Yebio, M.G., Obura, D., 1996. Effect of sea urchin reductions on algae, coral, and fish populations. Conservation Biology 10, 136–154.
Moschino, V., Marin, M.G., 2002. Spermiotoxicity and embryotoxicity of triphenyltin in the sea urchin *Paracentrotus lividus* Lmk. Applied Organometallic Chemistry 16, 175–181.
Moulin, L., Catarino, A.I., Claessens, T., Dubois, P., 2011. Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816). Marine Pollution Bulletin 62, 48–54.
Moureaux, C., Simon, J., Mannaerts, G., Catarino, A., Pernet, P., Dubois, P., 2011. Effects of field contamination by metals (Cd, Cu, Pb, Zn) on biometry and mechanics of echinoderm ossicles. Aquatic Toxicology 105, 698–707.
Nahon, S., Castro Porras, V.A., Pruski, A.M., Charles, F., 2009. Sensitivity to UV radiation in early life stages of the Mediterranean sea urchin *Sphaerechinus granularis* (Lamarck). Science of the Total Environment 407, 1892–1900.
ÓDonnell, M., Todgham, A., Sewell, M., Hammond, L., Ruggiero, K., Fangue, N., Zippay, M., Hofmann, G., 2010. Ocean acidification alters skeletogenesis and gene expression in larval sea urchins. Marine Ecology Progress Series 398, 157–171.
Palacín, C., Giribet, G., Carner, S., Dantart, L., Turon, X., 1998. Low densities of sea urchins influence the structure of algal assemblages in the western Mediterranean. Journal of Sea Research 39, 281–290.
Pearse, J.P., 2006. Ecological role of purple sea urchins. Science 314, 940–941.
Pennington, J.T., Strathmann, R.R., 1990. Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape. The Biological Bulletin 179, 121–133.
Pesando, D., Huitorel, P., Dolcini, V., Angelini, C., Guidetti, P., Falugi, C., 2003. Biological targets of neurotoxic pesticides analysed by alteration of developmental events in the Mediterranean sea urchin, *Paracentrotus lividus*. Marine Environmental Research 55, 39–57.
Peters, C., Becker, S., Noack, U., Pfitzner, S., Bülow, W., Barz, K., Ahlf, W., Berghahn, R., 2002. A marine bioassay test set to assess marine water and sediment quality-its need, the approach and first results. Ecotoxicology 11, 379–383.
Pillai, M.C., Vines, C.A., Wikramanayake, A.H., Cherr, G.N., 2003. Polycyclic aromatic hydrocarbons disrupt axial development in sea urchin embryos through a β-catenin dependent pathway. Toxicology 186, 93–108.
Quiniou, F., Guillou, M., Judas, A., 1999. Arrest and delay in embryonic development in sea urchin populations of the Bay of Brest (Brittany, France): link with environmental factors. Marine Pollution Bulletin 38, 401–406.
Radenac, G., Fichet, D., Miramand, P., 2001. Bioaccumulation and toxicity of four dissolved metals in *Paracentrotus lividus* sea-urchin embryo. Marine Environmental Research 51, 151–166.
R Development Core Team, 2008. R: A Language and Environment for Statistical Computing, vol. 1. Vienna Austria R Foundation for Statistical Computing, p. 7.
Rey-Asensio, A., Carballeira, C., Viana, I.G., Carballeira, A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioacumulación de microcontaminantes. In: Rey-Méndez, M.L.C., Fernández Casal, J., Guerra, A. (Eds.), Foro dos Recursos Mariños e da Acuicultura das Rías Galegas XIII. USC, O Grove, pp. 201–218.
Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., DeMarini, D.M., 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: A review and roadmap for research. Mutation Research/Reviews in Mutation Research 636, 178–242.
Ritz, C., Streibig, J.C., 2005. Bioassay analysis using R. Journal of Statistical Software 12.
Roccheri, M.C., Agnello, M., Bonaventura, R., Matranga, V., 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochemical and Biophysical Research Communications 321, 80–87.
Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. Aquatic Toxicology 71, 155–173.
Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicology and Environmental Safety 73, 491–499.
Salamanca, M., Fernández, N., Cesar, A., Antón, R., Lopez, P., DelValls, Á, 2009. Improved sea-urchin embryo bioassay for *in situ* evaluation of dredged material. Ecotoxicology 18, 1051–1057.
Sarà, G., 2007. A meta-analysis on the ecological effects of aquaculture on the water column: dissolved nutrients. Marine Environmental Research 63, 390–408.
Sbrilli, G., Bimbi, B., Cioni, F., Pagliai, L., Luchi, F., Lanciotti, E., 2005. Surface and ground waters characterization in Tuscany (Italy) by using algal bioassay and pesticide determinations: comparative evaluation of the results and hazard assessment of the pesticides impact on primary productivity. Chemosphere 58, 571–578.
Sharma, T., Ettensohn, C.A., 2010. Activation of the skeletogenic gene regulatory network in the early sea urchin embryo. Development 137, 1149–1157.
Siikavuopio, S.I., Dale, T., Christiansen, J.S., Nevermo, I., 2004. Effects of chronic nitrite exposure on gonad growth in green sea urchin *Strongylocentrotus droebachiensis*. Aquaculture 242, 357–363.
Soualili, D., Dubois, P., Gosselin, P., Pernet, P., Guillou, M., 2008. Assessment of seawater pollution by heavy metals in the neighbourhood of Algiers: use of the sea urchin, *Paracentrotus lividus*, as a bioindicator. ICES Journal of Marine Science: Journal du Conseil 65, 132–139.
Stalter, D., Magdeburg, A., Oehlmann, J., 2010. Comparative toxicity assessment of ozone and activated carbon treated sewage effluents using an *in vivo* test battery. Water Resources 44, 2610–2620.
Steevens, J.A., Slattery, M., Schlenk, D., Aryl, A., Benson, W.H., 1999. Effects of ultraviolet-B light and polyaromatic hydrocarbon exposure on sea urchin development and bacterial bioluminescence. Marine Environmental Research 48, 439–457.

Strathmann, R.R., Fenaux, L., Strathmann, M., 1992. Heterochronic developmental plasticity in larval sea urchins and its implicaions for evolution of nonfeeding larvae. Evolution 46, 972–986.
Tello, A., Corner, R.A., Telfer, T.C., 2010. How do land-based salmonid farms affect stream ecology? Environmental Pollution 158, 1147–1158.
Timourian, H., 1968. The effect of zinc on sea urchin morphogenesis. Journal of Experimental Zoology 169, 121–131.
Trieff, N.M., Romaña, L.A., Esposito, A., Oral, R., Quiniou, F., Iaccarino, M., Alcock, N., Ramanujam, V.M.S., Pagano, G., 1995. Effluent from bauxite factory induces developmental and reproductive damage in sea urchins. Archives of Environmental Contamination and Toxicology 28, 173–177.
Tuchmann-Duplessis, H., 1984. Drugs and other xenobiotics as teratogens. Pharmacology & Therapeutics 26, 273–344.
USEPA, 1994. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to West Coast Marine and Estuarine Organisms, third ed. United States Environmental Protection Agency, Cincinnati, p. 370.
Warnau, M., Temara, A., Jangoux, M., Dubois, P., Iaccarino, M., De Biase, A., Pagano, G., 1996. Spermiotoxicity and embryotoxicity of heavy metals in the echinoid *Paracentrotus lividus*. Environmental Toxicology and Chemistry 15, 1931–1936.
Wolska, L., Sagajdakow, A., Kuczynska, A., Namiesnik, J., 2007. Application of ecotoxicological studies in integrated environmental monitoring: possibilities and problems. TRAC Trends in Analytical Chemistry 26, 332–344.
Xu, X., Li, Y., Wang, Y., Wang, Y., 2011. Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay. Toxicology In Vitro 25, 294–300.

Arch Environ Contam Toxicol (2012) 63:249–261
DOI 10.1007/s00244-012-9769-0

Assessing the Toxicity of Chemical Compounds Associated With Land-Based Marine Fish Farms: The Sea Urchin Embryo Bioassay With *Paracentrotus lividus* and *Arbacia lixula*

C. Carballeira · M. R. De Orte · I. G. Viana · T. A. DelValls · A. Carballeira

Received: 1 July 2011 / Accepted: 9 April 2012 / Published online: 6 May 2012

Abstract In aquaculture, disinfection of facilities, prevention of fish diseases, and stimulation of fish growth are priority goals and the most important sources of toxic substances to the environment, together with excretory products from fish. In the present study, embryos of two species of sea urchin (*Paracentrotus lividus* and *Arbacia lixula*) were exposed to serial dilutions of six antibiotics (amoxicillin (AMOX), ampicillin, flumequine (FLU), oxytetracycline (OTC), streptomycin (ST), and sulfadiazine [SFD]) and two disinfectants (sodium hypochlorite (NaClO) and formaldehyde [CH_2O]). Alterations in larval development were studied, and the effective concentrations (ECs) were calculated to evaluate the toxicity of the substances. Both species showed similar sensitivities to all substances tested. Disinfectants (EC_{50} = 1.78 and 1.79 mg/l for CH_2O; EC_{50} = 10.15 and 11.1 mg/l for NaClO) were found to be more toxic than antibiotics. AMOX, OTC, and ST caused <20 % of alterations, even at the highest concentrations tested. FLU was the most toxic to *P. lividus* (EC_{50} = 31.0 mg/l) and SFD to *A. lixula* (EC_{50} = 12.7 mg/l). The sea urchin bioassay should be considered within toxicity assessment–monitoring plans because of the sensitivity of larvae to disinfectants.

Electronic supplementary material The online version of this article (doi:10.1007/s00244-012-9769-0) contains supplementary material, which is available to authorized users.

C. Carballeira (✉) · M. R. De Orte · T. A. DelValls
Departamento de Química Física, Facultad de Ciencias del Mar y Ambientales, Cátedra UNESCO/UNITWIN/WICOP, 11510 Puerto Real, Cádiz, Spain
e-mail: carlos.carballeira@uca.es

M. R. De Orte
e-mail: manoela.romano@uca.es

T. A. DelValls
e-mail: angel.valls@uca.es

I. G. Viana
Centro Costero de A Coruña, Instituto Español de Oceanografía, 15080 A Coruña, Spain
e-mail: inesgviana@gmail.com

A. Carballeira
Departamento de Ecología, Ecotoxicología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, Spain
e-mail: alejo.carballeira@usc.es

The discharge of organic waste and a wide range of chemicals is the main cause of the environmental impacts associated with intensive aquaculture (Douet et al. 2009; Burridge et al. 2010; Muñoz et al. 2010). The use of chemicals in aquaculture is essential for manipulating fish reproduction, increasing feeding efficiency, improving survival and growth rates, cleaning facilities, controlling pathogens and diseases, and decreasing transport stress (Fernandes et al. 2001; Huntington et al. 2006; Sapkota et al. 2008; Burridge et al. 2010). Few studies have addressed the environmental effects in coastal waters of chemicals commonly used in aquaculture (Huntington et al. 2006). Moreover, fish farm reports on disease occurrence, prescribed compounds, and quantities of compounds used are not available to the public (Sapkota et al. 2008; Burridge et al. 2010) because ecotoxicological information about chemotherapeutic and cleaning treatments are included in confidential technical reports, which can be difficult to obtain (Crane et al. 2007).

Antibiotics are mainly required to prevent and control diseases and to stimulate fish growth. It has been estimated that approximately 75 % of antibiotics are not absorbed in the gut by animals and are excreted as the parent compound

Table 1 Contribution of fish farming to contamination of the aquatic system: description of the main constituents of fish farming wastewaters

Type of waste		Origin	Frequency
Particulate	Feces	Food intake	Continuous
	No consumed food	Food intake	Continuous
Dissolved	Excretion products	Food intake	Continuous
	Pesticides	Preventing and removing fouling, parasites, etc.	Occasional
	Disinfectants	Cleaning and disinfection	Continuous
	Medicines (antibiotics and vaccines)	Disease control	Occasional
	Others (hormones, anesthetics, food additives, and structural materials)	Induce ovulation, pain insensitivity, coloring fish meat, facilitate degradation	Occasional
Biological	Pathogens transmissible to wildlife	Culture conditions (fish density, antibiotic resistance)	Occasional

Data from Carballeira et al. (2012b)

and its breakdown metabolites in waste (Chee-Sanford et al. 2009). Antibiotics can affect the biological diversity of phytoplanktonic and zooplanktonic communities (Holten-Lützhøft et al. 1999), and their use can lead to drug-resistant bacteria and transferable resistance genes in fish pathogens and other bacteria in the aquatic environment (Heuer et al. 2009).

Large quantities (10–30 mg/l) of disinfectants are used in intensive fish farming to disinfect sites, water, sediment, and equipment and sometimes to treat diseases (Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) 1997; Gräslund and Bengtsson 2001). No regulations exist regarding the use of disinfectants, and no information is available on the amounts used in the marine aquaculture industry, processing plants, or the food industry (Muñoz et al. 2010). Moreover, disinfectants contain surfactants, which may not be listed on the label (Burridge et al. 2010). The enormous dilution of aquaculture effluents, when they are discharged into aquatic systems, and the degradation of contaminants in the natural environment make it difficult to detect pollutants by chemical analysis of water samples (Pouliquen et al. 2007). Environmental assessment of chemicals in the marine environment therefore requires the development of highly specific and sensitive analytical procedures.

The sea urchin embryo development test has been found to be a suitable tool for assessing environmental pollution (Cesar et al. 2004; Bellas et al. 2005; Byrne et al. 2008; Mankiewicz-Boczek et al. 2008) and has been standardized by several national environmental agencies (United States Environmental Protection Agency (USEPA) 1994a; Environment Canada 1997). This test has been shown to be sensitive to metals (Kobayashi 1977; Kobayashi and Okamura 2004; Marín-Guirao et al. 2005; Kungolos et al. 2009; Caplat et al. 2010), organotin compounds (Kobayashi et al. 2008), radiation (Matranga et al. 2010; Bonaventura et al. 2005), and acidification (Byrne et al. 2010; Moulin et al. 2011), among other factors. It has also been found to be a valuable tool for evaluating complex chemical mixtures (in which additive, synergistic, and antagonistic effects may affect toxicity) that might be released into the marine environment (Xu et al. 2011). The sea urchin embryo development test has been proposed for inclusion in a battery of bioassays for monitoring the toxicity of effluents from land-based marine fish farms (LBMFFs; Carballeira et al. 2011a), which are characterized by the use of complex mixtures of substances.

The main objective of this study was to assess the validity of the sea urchin larval development test using the species *Arbacia lixula* and *Paracentrotus lividus* by evaluating the toxicity of fish farming–related chemicals. Larvae were exposed to six antibiotics [amoxicillin (AMOX), ampicillin (AMP), flumequine (FLU), oxytetracycline hydrochloride (OTC), streptomycin sulfate (ST), and sulfadiazine (SFD)], two disinfectants (sodium hypochlorite (NaClO) and formaldehyde [CH_2O]), and two reference toxicants (ammonium chloride (NH_4Cl) and zinc sulfate [$ZnSO_4$]). The target substances were selected because of their frequent use in several LBMFFs in Galicia (northwest Spain) (GESAMP 1997; Costello et al. 2001) (Table 1). The reference toxicants were those proposed by Cesar et al. (2002) for the embryo development toxicity test. Possible differences in the responses to the chemicals in the two species of sea urchin were analyzed to select the most sensitive bioindicator for the assessment of LBMFF wastes.

Materials and Methods

Dilution Preparation

Standard solutions of the selected antibiotics, disinfectants, and two reference toxicants were prepared with artificial seawater using Sigma-Aldrich reagents (Sigma-Aldrich Co. LLC, St. louis, USA) and borosilicate material at all times (Lera et al. 2006; USEPA 1995). The artificial seawater was prepared according to Lorenzo et al. (2002) and filtered (0.2 μm). Optimum ranges of salinity (32–34 ‰) and

pH (8.0 ± 0.4) were established for both *A. lixula* and *P. lividus*. These parameters were controlled at the beginning and the end of the test, so they could be ruled out as confounding factors (Carballeira et al. 2011c; Saco-Álvarez et al. 2010). The reagents were as follows: 99 % AMOX, 99 % AMP, 99 % FLU, 95 % OTC, 98 % ST, 99 % SFD, 10 % NaClO, 37 % CH_2O, 99.5 % NH_4Cl, and 99.9 % $ZnSO_4$.

Nominal dilutions of all of the chemicals were prepared by diluting the standard solutions with artificial seawater as follows. Dilutions of 100, 200, 500, and 750 mg/l were prepared for AMOX; 0.5, 1, 5, 10, 50, 100, 200, and 300 mg/l for AMP; 0.5, 1, 5, 10, 20, 50, 100, and 200 mg/l for FLU; 100, 200, 300, 500, and 750 mg/l for OTC; 0.5, 1, 5, 10, 50, 100, and 200 mg/l for ST; 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 mg/l for SFD; 0.25, 0.5, 1, 5, 10, 20, 35, and 50 mg/l for NaClO; 0.5, 1, 1.5, 3, 5, and 10 mg/l for CH_2O; 0.25, 0.5, 1, 2, 4, 8, 15, and 30 mg/l for NH_4Cl; and 0.01, 0.02, 0.05, 0.1 and 0.2 mg/l for $ZnSO_4$. Incubation borosilicate vials of 20 ml volume were filled with the different dilutions of chemicals or with artificial seawater only (control). The test was performed in quadruplicate.

Sea Urchin Embryo Development Bioassay

Adult sea urchins were collected from a clean intertidal zone at Getares (Algeciras Bay, southwest Spain). In the laboratory, organisms were acclimatized in a pool with clean aerated seawater in continual renewal at 15 °C for 10 days. Gametes were obtained by injecting 1 ml KCl (0.5 M) through the perioral membrane of three male and three female specimens. Sperms and eggs from adults were pooled separately in borosilicate beakers. The eggs were maintained in seawater, and sperm was kept in dry and cold conditions until in vitro fertilization. Gametes were observed under a microscope to check their maturity (spherical eggs and mobile sperm). The eggs were then transferred to a measuring cylinder containing artificial seawater. A few microliters of dry sperm were added to the egg suspension and stirred carefully to allow fertilization to take place (at least 95 % fertilized eggs). The density of fertilized eggs in the suspension was checked, and a volume of suspension containing approximately 400 fertilized eggs was placed in each incubation vial already filled with the chemical dilutions. The same methodology for in vitro fertilization was followed for both sea urchin species.

The tests with both of the species were conducted simultaneously. Vials were covered with a lid and incubated for 48 (*P. lividus*) and 72 h (*A. lixula*) under natural photoperiod (Fernández 2002), at 20 °C ± 0.7 and >85 % dissolved oxygen. These conditions precluded evaporation of the solutions and ensured that confounding factors would not interfere in larvae development (Saco-Álvarez et al. 2010), enabled complete development of embryos into pluteus larvae, and minimized background mortality and the duration of the tests (Fernández 2002). After the incubation period, larval development was stopped, and larvae were fixed by adding two drops of 40 % CH_2O. The end point was the failure of embryogenesis, which was expressed as the percentage of abnormal larvae. A complete description of the criterium by which to distinguish altered and normal larvae can be found in Carballeira et al. (2012a).

Statistical Analysis

Differences Among Dilutions

Significant differences between the percentage of abnormal embryos obtained after exposure to artificial seawater (control) and to the test dilutions were determined for each chemical by one-way analysis of variance (ANOVA), followed by multiple comparisons with Dunett and Tukey tests. The differences were classified according to their statistical significance as follows: a ($p < 0.001$), b ($p < 0.01$), and c ($p < 0.05$). SPSS statistical software (Statistical Package for the Social Sciences, version 17.0, International Business Machines Corporation, Massachusetts, USA) was used for data analysis.

Dose–Response Curves and EC Values

R software (The R foundation for Statistical computing, Vienna, Austria) with the drc add-on package was used to calculate the best-fitted dose–response curves for each chemical and each species. Data for each chemical and each species were adjusted to the most suitable parametric of a series of nonlinear regression models, according to the maximum log likelihood value, Akaike's information criterion, the estimated residual variance, and the p value from a lack-of-fit test (Ritz and Streibig 2005; R Development Core Team 2008). Then the software calculated and provided the effective concentrations (ECs) for the different chemicals tested and their respective SD. The effective concentration (ECx) of a substance is defined as the concentration that causes a defined magnitude (x) of response in a given organism.

Species Sensitivity

Differences in the sensitivity to chemicals between the two sea urchin species, according to the failure of embryological success and the EC values, were analyzed by nonparametric Mann–Whitney test. Differences were considered significant at 95 % ($p < 0.05$). Statistical software (SPSS version 17.0) was used to perform the analyses.

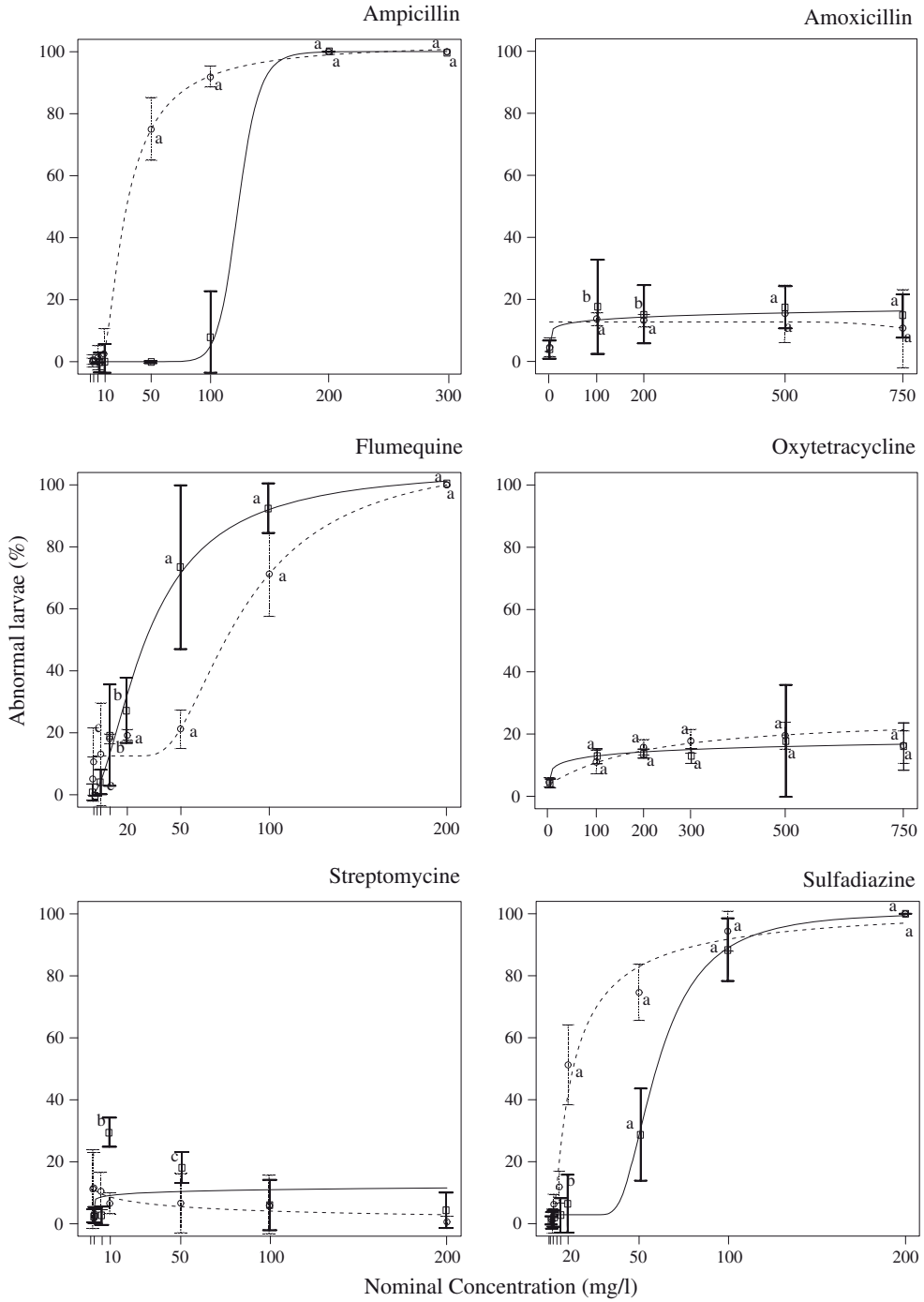


Fig. 1 Dose–response curves for each antibiotic obtained for *P. lividus* (*solid line* and *squares*) and *A. lixula* (*dashed line* and *circles*). EC values are based on nominal concentrations at the beginning of the toxicity test. Significant differences between dilutions and control are represented as follows: *a* ($p < 0.001$), *b* ($p < 0.01$), and *c* ($p < 0.05$)

Results

Toxic Responses

The dose–response curves obtained for the antibiotics, disinfectants, and reference toxicants, according to the embryological failure of *P. lividus* and *A. lixula*, are shown in Figs. 1, 2. The EC values are listed in Table 2. Most data from the bioassay with *P. lividus* could be fitted to the four-parameter log-logistic model. Some data from the bioassay with *A. lixula* were fitted to the four parameter Weibull model, and other dose–response curves were also calculated using the four parameter log-logistic model (Ritz and Streibig 2005).

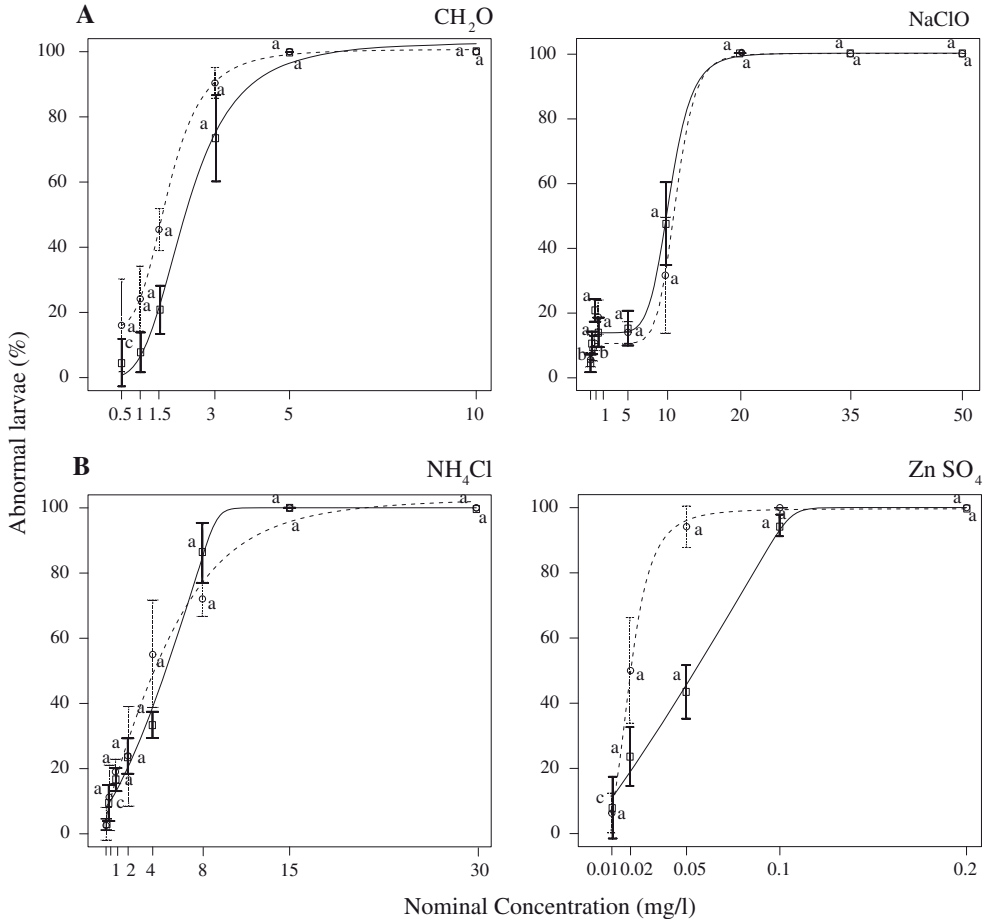


Fig. 2 Dose–response curves for disinfectants (**a**), ammonia (**b**), and reference substances (**c**) obtained for *P. lividus* (*solid line* and *squares*) and *A. lixula* (*dashed line* and *circles*). EC values are based on nominal concentrations at the beginning of the toxicity test. Significant differences between dilutions and reference are represented as follows: *a* ($p < 0.001$), *b* ($p < 0.01$), and *c* ($p < 0.05$)

Antibiotics

The percentages of abnormal larvae of both species after exposure to AMOX, OTC, and ST were significantly different ($p < 0.05$) from the controls in all of the dilutions, but they remained <20 %, even at the highest antibiotic concentrations tested (750, 750, and 200 mg/l, respectively) (Fig. 1). Because the maximum and minimum observed responses must be smaller or larger than the EC_x level to be calculated (Ritz and Streibig 2012), it was not possible to calculate the EC_{20} and EC_{50} values for these chemicals (Table 2). The percentages of abnormal larvae of the two species were significantly different ($p < 0.05$) from the control in all of the dilutions, and they increased greatly with the concentrations of AMP, FLU, and SFD; complete larval alteration was reached at a concentration of 200 mg/l (Fig. 1). When comparing the various EC values of the different antibiotics (Table 2), results from the *A. lixula* test clearly showed the highest EC values obtained with AMOX, OTC, and ST (EC_{10} = 1,276 and 85.1 and EC_5 = 64.3 mg/l, respectively), whereas lowest EC values were obtained with AMP, FLU, and SFD (EC_{50} = 2.93, 75.4, and 12.2 mg/l, respectively). Results from the *P. lividus* test were more variable, but all of the EC values were lower than for *A. lixula* in the case of FLU. None of the antibiotics were associated with a particular larval deformation in any of the species.

Disinfectants

The percentages of abnormal larvae of *P. lividus* and *A. lixula* increased along with the concentrations of CH_2O and NaClO, and values >50 % were reached with the 1.7 and 10 mg/l dilutions, respectively (Fig. 2a). Embryogenesis failed in a significantly different way from the control in all of the dilutions.

The EC values for CH_2O were among the three lowest of all of the test substances for both species (Table 2). Regarding NaClO, only the EC_{20} and the EC_{50} were able to be determined, and the values were intermediate compared

Table 2 EC values (mg/l) obtained with the sea urchin embryo development test performed with *P. lividus* and *A. lixula*[a]

Chemical	*P. lividus*					*A. lixula*					Significance
	EC_5	EC_{10}	EC_{20}	EC_{50}	*p*	EC_5	EC_{10}	EC_{20}	EC_{50}	*p*	
Antibiotics											
AMOX	**26.5 (±34.67)**	108 (±42.66)	ND	ND	0.1578	898 (±560.30)	**1,276 (±2,144.41)**	ND	ND	4.35E-04	0.515
AMP	**100 (±108.29)**	**117 (±131.82)**	**136 (±160.62)**	**168 (±210.02)**	1.00	11.4 (±1.07)	13.5 (±1.11)	17.1 (±1.17)	29.3 (±1.56)	0.9099	0.047*
FLU	5 (±1.38)	8.04 (±1.56)	13.4 (±2.07)	31.01 (±7.89)	0.5471	ND	ND	48.6 (±4.13)	75.4 (±5.63)	0.0005	0.844
OTC	ND	**21.8 (±112.21)**	**	**	0.2718	**12.7 (±13.60)**	85.1 (±51.10)	**533 (±967.92)**	ND	0.1428	0.232
ST	0.02 (±0.017)	29.8 (±16.67)	**	**	0.1279	**64.3 (±83.40)**	**3.90 (±13.63)**	ND	ND	0.7973	0.168
SFD	36.7 (±1.20)	41 (±1.02)	46.2 (±0.83)	59.76 (±1.23)	0.2962	2.46 (±0.43)	3.32 (±0.44)	4.98 (±0.46)	12.7 (±1.93)	5.85E-03	0.219
Disinfectants											
CH_2O	0.881 (±0.61)	1.23 (±0.32)	1.44 (±0.07)	1.78 (±0.40)	0.0448	0.951 (±0.26)	1.23 (±0.15)	1.44 (±0.04)	1.79 (±0.20)	0.8325	0.246
NaClO	ND	ND	7.40 (±0.96)	10.15 (±0.18)	5.71E-06	ND	ND	8.96 (±0.68)	11.1 (±0.79)	0.0017	0.812
Reference toxicants											
NH_4Cl	ND	0.573 (±0.19)	1.97 (±0.34)	5.04 (±0.30)	5.90E-04	0.23 (ND)	0.54 (ND)	1.29 (ND)	4.17 (±0.40)	0.0079	0.868
$ZnSO_4$	0.002 (±0.0011)	0.009 (±0.0032)	0.021 (±0.0048)	0.054 (±0.0048)	0.0107	0.009 (±0.0006)	0.011 (±0.0006)	0.014 (±0.0005)	0.021 (±0.0004)	0.6873	0.301

ND not determined

[a] Results of Mann–Whitney test for the comparison of relative sensitivities to the tested chemicals between the two species considered are also included. The *p* value of each dose–response curve model is also shown. EC values are based on nominal concentrations at the beginning of the test. Those EC values whose SE is greater than the EC value are *bolded*

* $p < 0.05$

** Above solubility level

Table 3 ECs of reference toxicants obtained in previous studies with the sea urchin embryo development test

Toxicant	Species	End point	Value (mg/l)	Reference
Ammonium chloride	*A. lixula*	EC_{25}/EC_{50}	1.71/2.48	Cesar et al. (2002)
	P. lividus	EC_{25}/EC_{50}	1.95/2.71	
	Sphaerechinus granularis	EC_{25}/EC_{50}	1.87/2.24	
	A. lixula	EC_{25}/EC_{50}	1.70/2.56	Cesar et al. (2004)
	P. lividus	EC_{25}/EC_{50}	1.8/2.72	
	S. granularis	EC_{25}/EC_{50}	1.71/2.30	
Zinc sulfate	*Sterechinus neumayeri*	EC_{50} (20–23 d)	0. 326	King and Riddle (2001)
	A. lixula	EC_{50}	0.01–0.100	
	P. lividus	EC_{50}	<0.033	
	Hemicentrotus pulcherrinus	EC_{50}	0.010–0.020	
	Diadema setosum	EC_{50}	0.010–0.020	
	P. lividus	EC_{50}	0.8–1.0	Caplat et al. (2010)
	A. lixula	EC_{25}/EC_{50}	0.03/0.05	Cesar et al. (2002)
	P. lividus	EC_{25}/EC_{50}	0.03/0.05	
	S. granularis	EC_{25}/EC_{50}	0.03/0.05	
	A. lixula	EC_{25}/EC_{50}	0.03/0.04	Cesar et al. (2004)
	P. lividus	EC_{25}/EC_{50}	0.03/0.05	
	S. granularis	EC_{25}/EC_{50}	0.03/0.06	

with those obtained for the other chemicals (Table 2). The disinfectants were not associated with any particular larval deformation in either of the species.

Reference Toxicants

The ECs of the reference toxicants (Table 2), calculated from the best-fit dose–response curves (Fig. 2b), were consistent with results reported by environmental agencies (Environment Canada 1997; CETESB 1999) and by other investigators (Table 3). More than 80 % of larvae exposed to solutions of 2 mg/l of NH_4Cl had a crossed tip.

Species Sensitivity

P. lividus and *A. lixula* displayed similar degrees of sensitivity to the tested chemicals, including the reference toxicants (Table 2), except for AMP. *A. lixula* was five times more sensitive to AMP than *P. lividus*. The responses of the sea urchins to FLU, NaClO, and NH_4Cl were similar (>75 %).

Discussion

General Approach

Bioassays using sea urchins have been recommended for inclusion in an integrated approach to the pharmacological evaluation of traditional *materia medica* (Kyerematen and Ogunlana 1987). Here, we will determine if they should be included in monitoring plans for LBMFFs.

Intensive LBMFFs are usually located at highly exposed coasts with strong hydrodinamics. Because of this, wastewaters are rapidly diluted and dispersed, difficulting the determination of contaminants by conventional chemical analysis. Rey-Asensio et al. (2010) measured metals, pesticides, and antibiotics in the water column and biological samples from the vicinity of a series of LBMFFs. They concluded that chemical determinations, except for the ^{15}N signal, were not a suitable method to monitor the impact of these farms because the concentrations of all contaminants were lower than the detection limit in the water column. Some low concentrations were found in anemoneae and algae but the samples did not represent a contamination gradient. However, biological effects have been identified in different marine species exposed to LBMFF effluents under laboratory conditions (Carballeira et al. 2011a). Despite the low concentration of contaminants in the LBMFF surroundings, biological damage has been observed in native biota (Carballeira et al. 2011b), indicating that the dilution of the effluents does not preclude damage to the ecosystem. Therefore, toxicity tests may be a more effective method to monitor intensive aquaculture. To implement a useful battery of bioassays for LBMFF monitoring, the sensitivity of different organisms to aquaculture-related contaminants should be first assessed.

In the present work, the suitability of the sea urchin larval development test to evaluate the toxicity chemicals

related with fish farming was evaluated. Larvae of the species *P. lividus* and *A. lixula* were exposed to a series of nominal concentrations of antibiotics and disinfectants. Two reference substances were also included to determine the acceptability of the test.

The toxicity of metals and some organic pollutants decreases with increasing salinity (Hall and Anderson 1995). In this study, the salinity of all dilutions was held constant (33 ‰) to determine the potential toxicity of aquaculture effluents on the surroundings of LBMMFs. However, because of the lack of information on the toxicity of tested chemicals, end point values were compared with those obtained at different salinities in other studies.

The toxicity of other compounds depends on pH, temperature, and salinity (Bell et al. 2007) as well as the photodegradation rate (Yuan et al. 2011). In this study, pH, temperature, and salinity of all dilutions were controlled and kept within the optimal ranges for the species, and a natural photoperiod was applied to resemble natural photodegradation. These conditions were the same for both species; therefore, exposure characteristics were similar, and interspecies comparisons could be conducted to enable the selection of the most sensitive species.

Antibiotics

The results of the present study showed that at concentrations >100 mg/l, some antibiotics, such as AMOX, OTC, and ST, may improve the development of sea urchin larvae, whereas others, such as AMP, FLU, and SFD, may inhibit sea urchin larva development at a concentration of approximately 100 mg/l. Administration of antibiotics has been recognized as a way of improving the survival of marine larvae (Verschuere et al. 2000). In fact, antibiotics can be used in aquaculture as growth stimulators rather than measures for disease prevention and can be included in commercial feeds (Flaherty et al. 2000). Nevertheless, chemotherapeutants may also be toxic depending on the therapeutic class, the administration period, or the dose used (National Research Council 1993). Antibiotics are designed to inhibit the growth of pathogenic bacteria and kill them by selective inhibition of the synthesis of the cell wall and other membranes, macromolecular synthesis, or enzyme activity in prokaryotic cells. As a result of these selective traits, they show low toxicity in higher organisms (Nikaido 2009). The log Kow (octanol–water partition coefficient) values of most antibiotics are <5, indicating that they are not particularly hydrophobic (Tolls 2001). Antibiotics mainly act on prokaryotic organisms, which have also been shown to be more sensitive (Halling-Sørensen 2001). Thus, in theory, antibiotics may be rapidly dispersed by dilution and may be better detected using bacterial bioassays rather than using bioassays with other organisms. Nevertheless, this study has shown the validity of the embryo development test in assessing the toxicity of at least three of the six antibiotics tested: AMP, FLU, and SFD. Moreover, several research studies have shown that tests with bacteria may not be the most appropriate for monitoring antibiotics in the environment (see later text).

This study is important because of the lack of research on the use of the sea urchin bioassay to assess the toxicity of antibiotics. Most studies on the deleterious effects of antibiotics on marine organisms have focused on marine bacteria, green algae, and crustaceans. As already mentioned, the sensitivity of the two sea urchin species used, *P. lividus* and *A. lixula*, varied depending on the antibiotic considered. The larval development of both species was significantly affected by AMP, FLU, and SFD. Conversely, larvae of both species were found to be resistant to AMOX, OTC, and ST.

AMOX

AMOX has been tested in diverse bioassays with bacteria, algae, invertebrates, and fish and in most cases has been found to be associated with EC_{50} values >1,000 mg/l, indicating low toxicity for all tested taxa (Eguchi et al. 2004; Holten-Lützhøft et al. 1999; Park and Choi 2008). However, low tolerances were also reported for two freshwater organisms: blue-green microalgae *Synechococcus lepoliensis* (EC_{50} = 0.002 mg/l) and the cyanobacteria *Microcystis aeruginosa* (EC_{50} = 0.0037 mg/l) (Andreozzi et al. 2006; Holten-Lützhøft et al. 1999). This fact indicates a certain specificity of AMOX because it seems to affect mainly bacteria, which are the target.

OTC

The toxicity of OTC has been previously assessed with marine organisms and displayed high variability among species. Freshwater (e.g., green algae *S. capricornutum*, rotifer *Brachionus calyciflorus*, and cladoceran *Ceriodaphnia dubia*) and marine species [e.g., shrimp *Penaeus vannamei* (Boxall et al. 2004; Isidori et al. 2005)] as well as green algae *Rhodomonas salina* (Holten-Lützhøft et al. 1999) were found to be more sensitive to OTC (EC_{50} = 0.061–0.18 mg/l) than marine bacteria [e.g., *Vibrio fischeri* (Isidori et al. 2005; Lalumera et al. 2004; Park and Choi 2008)], sea urchins (e.g., *Psammechinus miliaris* [Campbell et al. 2001]), or larvae of the marine fish *Morone saxatilis* (Boxall et al. 2004) ($EC_{50} \geq 60$ mg/l). However, in the case of bacteria, the apparently low sensitivity may be due to the short exposure time used in the toxicity test rather than to a real lack of sensitivity (Thomulka et al. 1993; Backhaus and Grimme 1999; van der Grinten et al. 2010).

The present results were consistent with those reported by other investigators who considered ST as nontoxic to sea urchins. In fact, Lera and Pellegrini (2006) used ST to decrease marine bacterial contamination in the culture and cryogenic storage of *P. lividus* eggs. Nevertheless, some studies on antimicrobial potency have shown that ST and OTC retained greater potency longer than other antibiotics in aerobic and anaerobic experiments, respectively (Halling-Sørensen et al. 2003). Some of the few studies of this antibiotic showed that the freshwater crustacean *Daphnia magna* [$EC_{50} > 480$ mg/l (Wollenberger et al. 2000)] was less sensitive than *M. aeruginosa* ($EC_{50} = 0.034$ mg/l [van der Grinten et al. 2010]), sludge bacteria [$EC_{50} = 0.47$ mg/l (Halling-Sørensen 2001)], and marine *V. harveyi* [$EC_{50} = 19$ mg/l (Thomulka et al. 1993)]. In contrast, *V. fischeri* was found to be highly resistant to ST, and the percentage of bioluminescence inhibition was maintained at <15 % at ST concentrations of 250 mg/l (Inés G. Viana, unpublished results).

AMP

The toxicity of AMP has been tested with *V. fischeri* (Backhaus and Grimme 1999; Park and Choi 2008), the freshwater cladocerans *D. magna* and *Moina macrocopa*, and the amphidromous fish *Oryzias latipes* (Park and Choi 2008). High tolerance was reported for all species, with $EC_{50} > 160$ mg/l. Park and Choi (2008) were not able to calculate the toxic end point (EC_{50}) of AMP for different trophic levels (bacteria, cladocera, and fish), even at the maximum concentrations tested (1,000 mg/l). The only way of detecting toxic concentrations of AMP < 1,000 mg/l was to extend the length of the bioluminescence test with *V. fischeri* to 24 h with an EC_{50} value of 163 mg/l (Backhaus and Grimme 1999). The sea urchin test thus appeared to be more sensitive to this antibiotic ($EC_{50} = 29.3$ mg/l).

FLU

In the present study, FLU was one of the most toxic antibiotics for *P. lividus* and *A. lixula*. The sensitivities of *V. fischeri* (Backhaus et al. 2000; Hernando et al. 2007; Lalumera et al. 2004), *M. aeruginosa* (Holten-Lützhøft et al. 1999; van der Grinten et al. 2010), and the microalga *R. salina* (Holten-Lützhøft et al. 1999) to this compound were found to be even greater ($EC_{50} = 0.198$–19 mg/l, 0.159 mg/l, and 18 mg/l, respectively).

SFD

Of the antibiotics assayed in this study, SFD was one of the most toxic; however, and again, other freshwater species, such as *M. aeruginosa* ($EC_{50} = 0.135$ mg/l) (Holten-Lützhøft et al. 1999), *S. capricornutum*, and *Chlorella vulgaris* ($EC_{50} = 2.2$ mg/l) (Eguchi et al. 2004) exhibited greater sensitivity than the sea urchin species tested here.

The results of the evaluation of the potential impact of antibiotics appear to depend on the sensitivity of the species used in the toxicity tests. *M. aeruginosa* appears to be the best candidate for assessing the toxicity of AMOX, FLU, and SFD because of its high sensitivity compared with other organisms tested. Crustacean and green microalgae appear appropriate for monitoring OTC, whereas bacteria, except *V. fischeri*, are appropriate for ST and sea urchins for AMP.

According to the REACH regulations (Registration, Evaluation, Authorization and Restriction of Chemicals), a substance is considered persistent, bioaccumulative, and toxic (PBT) when its half-life is >40 days in fresh or estuarine water, the bioaccumulation factor (BCF) is >2,000 l/kg, and the minimum no observed–effect concentration is <0.01 mg/l. The potential bioavailability of these antibiotics is variable, highlighting the high solubility of ST, AMOX, and OTC, although they are less soluble in seawater; FLU appears to be the most dangerous because it is more likely to persist in food chains (as indicated by the K_{ow} value) (Holten-Lützhøft et al. 1999; Beausse 2004; Chee-Sanford et al. 2009). In addition, although these antibiotics have shown relatively low toxicity, the half-life is >40 days, which may lead to acquisition of bacterial resistance.

Disinfectants

CH_2O

CH_2O is used worldwide as antifungal agent (piscicide) in the control of ectoparasites and is also used to remove ammonia from shrimp ponds (Gräslund and Bengtsson 2001). Regulations for maximum permissible levels of disinfection by-products have been established by the World Health Organization (WHO) as 0.9 mg/l for CH_2O (WHO 1993). The EC_5 values obtained for *P. lividus* and *A. lixula* are close to the concentration established by the WHO. Taking into account that the EC_5 is not even considered as an effect indicator, the WHO regulations are sufficiently restrictive and safeguard the integrity of sea urchins. There are no previous toxicity studies of CH_2O involving the sea urchin embryo development test, even although it is used to block larval development in the sea urchin bioassay and thus is known to be highly toxic to these organisms. The toxicity of this chemical has mainly been tested in bacteria, crustaceans, fish, and macroalgae. The WHO threshold appears appropriate for *V. fischeri* ($EC_{50} = 8.1$ mg/l [Ricco et al. 2004]) and for the

freshwater fish *Oncorhynchus mykiss* (EC_{50} = 50 mg/l) and *Danio rerio* (EC_{50} = 9 mg/l) (Eternal Technology Corporation 2004). In contrast, the WHO restriction for CH_2O may not be sufficient for *D. magna* (EC_{50} = 42 μg/l), *C. dubia* (EC_{50} = 12.9 μg/l), the crustaceans *Artemia* sp. (EC_{50} = 1.17 μg/l), the shrimp *Pennaeus stylirostris* (EC_{50} = 2.7 μg/l), the freshwater crustacean *Cypridopsis* sp. (EC_{50} = 2.56 μg/l), and numerous shrimps and crabs (EC_{50} = 7.6-633 μg/l) (Eternal Technology Corporation 2011) or the marine brown algae *Phyllospora comosa* (EC_{50} = 0.1 mg/l [Burridge et al. 2010]).

NaClO

NaClO is the most common disinfectant worldwide. In solution, it is quickly transformed, and the by-products generated may produce negative effects on the environment because they can react with different organic substances and result in high concentrations of halogenated hydrocarbons, which may be persistent (Flaherty et al. 2000; Gräslund and Bengtsson 2001). *P. lividus* and *A. lixula* showed intermediate sensitivity to NaClO compared with other species. *V. fischeri* (EC_{50} = 0.061) (Thomulka et al. 1993) and the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus variegatus* (EC_{50} = 0.064–0.7 mg/l) (Muchmore and Epel 1973; Sano et al. 2004) appeared to be particularly sensitive to this chemical.

NaClO decomposes on exposure to heat, ultraviolet light, and contaminants as follows (Corporation Black and Veatch 2010): $3NaOCl = 2NaCl + NaClO_3$. Chlorate (ClO_3^-) was found to be particularly toxic to the marine brown algae *Ectocarpus variabilis* (EC_{50} = 0.015 mg/l), which exhibited a sensitivity between 50 and 1,000 times greater than other species, such as the green microalgae *S. capricornutum* and the fungus *Trichoderma hamatum* (Van Wijk et al. 1998). The absence of common brown macroalgae (*Fucus sp.*) in the surroundings of sewage water outfalls confirms these results (Borowitzka 1972; Carballeira et al. 2012b; Littler and Murray 1975). Chlorine is also found in drinking water disinfected with chlorine dioxide and NaClO (Corporation Black and Veatch 2010) and is known to be highly toxic to *Daphnia* sp., with an EC_{50} from 0.005 to 0.15 mg/l (USEPA 1994b).

Reference Toxicants

The toxicity values of reference toxicants from both species were consistent with those found in previous studies with the same and other species. This indicated that the larval toxicity test was performed successfully and demonstrated the high reproducibility of this bioassay.

In solution, NH_4Cl dissociates and establishes an equilibrium unionized–ionized ammonia, which depends on pH, temperature, and salinity (Bell et al. 2007), and it also influences ammonia toxicity (Körner et al. 2001). In the present work, these parameters were controlled and kept within the optimal ranges for the species. Therefore, they did not vary significantly during the bioassay, and the equilibrium was not expected to vary either. Moreover, when the nominal concentration of total ammonia is known, the characteristics of the equilibrium at certain values of pH, temperature, and salinity can be assessed using the empirically determined equation by Bell et al. (2007).

Ammonia is the main end product of protein metabolism in teleosts (Altinok and Grizzle 2004), and it should be considered within the environmental monitoring plans of aquaculture farms because it can indicate the risk of eutrophication (Constable et al. 2003). It also represents a confounding factor when interpreting sea urchin toxicity tests results (Carballeira et al. 2011c; Carr et al. 2006; SacoÁlvarez et al. 2010) and in masking the toxicity of chemical mixtures (Arizzi Novelli et al. 2003; Carr et al. 2006; Losso et al. 2007).

In the present work, microscopic examination of larval skeletons showed that crossed tip was the prevailing skeletal malformation in solutions of NH_4Cl at concentrations of 1 and 2 mg/l. Carballeira et al. (2012a) studied the toxicity of effluents from land-based marine fish farms by analyzing specific malformations of the larval skeleton. Some effluents were characterized by a particular skeletal abnormality, and only one was associated with crossed tip as the dominant abnormality. Comparing the results of the present study with those of the latter study (Carballeira et al. 2012a), we can conclude that effluents from LBMFFs may contain substances other than NH_4Cl, or mixtures of substances, that cause diverse skeletal malformations.

Sensitivity Differences Between Species

There were no significant differences between the sensitivities of *P. lividus* and *A. lixula* to the tested compounds (except AMP). Previous studies have also shown that different sea urchin species display similar sensitivities when exposed to sediment and effluent samples, reference toxicants, metals, and mixtures of metals (Cesar et al. 2002, 2004; Xu et al. 2011; Carballeira et al. 2011a).

The absence of particularly sensitive sea urchin species in this and previous studies may offer the possibility to conduct toxicity tests with either species when the target species is not present as well as process and compare the results without distinction. However, further research is needed to confirm the validity of this hypothetical approach. More studies involving a greater number of species should be conducted, and the end point should be clearly established and strictly the same for all of the species (King and Riddle 2001).

Conclusion

The prolonged and indiscriminate use of chemicals in aquaculture is well documented, although little is known about their environmental effects. Safety for human consumption must be ensured and impacts on the environment minimized because aquaculture activity is expected to increase in coming years.

Bacterial toxicity tests are suitable for measuring the toxic effects of antibiotics because of their sensitivity to these antimicrobial agents. However, bacteria have shown lower sensitivity to disinfectants and metallic compounds than sea urchins and other species of different phyla. Therefore, a battery of tests, including one species of bacteria, one species of microalgae, and one species of sea urchin, is recommended for full screening of the toxic effects of substances and monitoring LBMFF surroundings in a rapid and cost-effective manner.

The application and comparison of more than one model for fitting the data allows selection of the model that best fits the data, thus enabling calculation of more EC values. Finally, the results of this study indicate that the sea urchin species *P. lividus* and *A. lixula* are equally sensitive to different types of chemicals (antibiotics, disinfectants, and reference toxicants), and so can be used indistinctly.

Acknowledgments This study was partly funded by the National Marine Aquaculture Plan. JACUMAR Project (2008): "Selection of indicators, determination of reference values, design of programs, protocols and measures for environmental studies in aquaculture (INDAQUA)." C. Carballeira is grateful for financial support from the University of Cadiz Predoctoral Fellowship Programme (Spain).

References

Altinok I, Grizzle JM (2004) Excretion of ammonia and urea by phylogenetically diverse fish species in low salinities. Aquaculture 238(1–4):499–507

Andreozzi R, Canterino M, Giudice RL, Marotta R, Pinto G, Pollio A (2006) Lincomycin solar photodegradation, algal toxicity and removal from wastewaters by means of ozonation. Water Res 40(3):630–638

Arizzi Novelli A, Picone M, Losso C, Volpi Ghirardini AM (2003) Ammonia as confounding factor in toxicity tests with the sea urchin *Paracentrotus lividus* (Lmk). Toxicol Environ Chem 85(4):183–191

Backhaus T, Grimme LH (1999) The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. Chemosphere 38(14):3291–3301

Backhaus T, Scholze M, Grimme LH (2000) The single substance and mixture toxicity of quinolones to the bioluminescent bacterium *Vibrio fischeri*. Aquat Toxicol 49(1–2):49–61

Beausse J (2004) Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. Trends Analyt Chem 23(10):753–761

Bell TG, Johnson MT, Jickells TD, Liss PS (2007) Ammonia/ammonium dissociation coefficient in seawater: a significant numerical correction. Environ Chem 4(3):183–186

Bellas J, Beiras R, Mariño-Balsa J, Fernández N (2005) Toxicity of organic compounds to marine invertebrate embryos and larvae: a comparison between the sea urchin embryogenesis bioassay and alternative test species. Ecotoxicology 14(3):337–353

Bonaventura R, Poma V, Costa C, Matranga V (2005) UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochem Biophys Res Commun 328(1):150–157

Borowitzka MA (1972) Intertidal algal species diversity and the effect of pollution. Aust J Mar Fresh Res 23(2):73–84

Boxall ABA, Fogg LA, Blackwell PA, Blackwell P, Kay P, Pemberton EJ et al (2004) Veterinary medicines in the environment. In: Albert LA, Gerba CP, Giesy J et al (eds) Reviews of environmental contamination and toxicology, vol 180. Springer, New York, p 91

Burridge L, Weis JS, Cabello F, Pizarro J, Bostick K (2010) Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. Aquaculture 306(1–4):7–23

Byrne M, Oakes D, Pollak J, Laginestra E (2008) Toxicity of landfill leachate to sea urchin development with a focus on ammonia. Cell Biol Toxicol 24(6):503–512

Byrne M, Soars N, Selvakumaraswamy P, Dworjanyn SA, Davis AR (2010) Sea urchin fertilization in a warm, acidified and high pCO_2 ocean across a range of sperm densities. Mar Environ Res 69(4):234–239

Campbell DA, Pantazis P, Kelly MS (2001) Impact and residence time of oxytetracycline in the sea urchin, *Psammechinus miliaris*, a potential aquaculture species. Aquaculture 202(1–2):73–87

Caplat C, Oral R, Mahaut ML, Mao A, Barillier D, Guida M et al (2010) Comparative toxicities of aluminum and zinc from sacrificial anodes or from sulfate salt in sea urchin embryos and sperm. Ecotoxicol Environ Saf 73:1138–1143

Carballeira C, De Orte MR, VianaI IG, Carballeira A (2011a) Implementation of a minimal biological test set for assessment of ecotoxic effect of effluents from land-based fish farms. Ecotox Environ Saf 78:148–161

Carballeira C, Espinosa J, Carballeira A (2011b) Linking N^{15} and histopathological effects in molluscs exposed in situ to effluents from land-based marine fish farms. Mar Poll Bull 62(12):2633–2641

Carballeira C, Martín-Díaz ML, DelValls TA (2011c) Optimization of fertilization and larval development toxicity tests using two marine sea urchin species. Study of salinity influence. Mar Environ Res 72(4):196–203

Carballeira C, Martín-Díaz L, DelValls TA (2012a) Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents. Mar Environ Res. doi:10.1016/j.marenvres.2012.01.001

Carballeira C, Martín-Díaz ML, DelValls TA, Carballeira A (2012b) Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas. J Environ Monit. doi:10.1039/c2em10839a

Carr R, Biedenbach J, Nipper M (2006) Influence of potentially confounding factors on sea urchin porewater toxicity tests. Arch Environ Contam Toxicol 51(4):573–579

Cesar A, Marín-Guirao L, Vita R, Marín A (2002) Sensitivity of mediterranean amphipods and sea urchins to reference toxicants. Cienc Mar 28(4):407–417

Cesar A, Marín A, Marín-Guirao L, Vita I (2004) Amphipod and sea urchin tests to assess the toxicity of mediterranean sediments: the case of Portmán Bay. Sci Mar 68(1):205–213

Chee-Sanford JC, Mackie RI, Koike S, Krapac IG, Lin YF, Yannarell AC et al (2009) Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J Environ Qual 38(3):1086–1108

Centro Tecnológico de Saneamento Básico (1999) Metodo de ensaio: Agua do mar—Teste de toxicidade cronica de curta duracao com *Lytechinus variegatus*, Lamark,1816 (Echinodermata: Echinoidea). CETESB do Estado de Sao Paulo, São Paulo, Brasil

Constable M, Charlton M, Jensen F, McDonald K, Craig G, Taylor KW (2003) An ecological risk assessment of ammonia in the aquatic environment. Hum Ecol Risk Assess 9(2):527–548

Corporation Black and Veatch (2010) White's handbook of chlorination and alternative disinfectants, 5th edn. Wiley, New Jersey, p 1062

Costello MJ, Grant A, Davies IM, Cecchini S, Papoutsoglou S, Quigley D et al (2001) The control of chemicals used in aquaculture in Europe. J Appl Ichthyol 17(4):173–180

Crane M, Burton GA, Culp JM, Greenberg MS, Munkittrick KR, Ribeiro R et al (2007) Review of aquatic in situ approaches for stressor and effect diagnosis. Integr Environ Assess Manage 3(2):234–245

Douet DG, Le Bris H, Giraud E (2009) Environmental aspects of drug and chemical use in aquaculture: an overview. Options Méditerranéennes 86:105–126

Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K et al (2004) Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57(11): 1733–1738

Environment Canada (1997) Biological test method: fertilization assay using echinoids (sea urchins and sand dollars). Method development and applications. Environmental Technology Center, Ottawa

Eternal Technology Corporation (2004) Material safety data sheet of formaldehyde. Eternal Technology Corporation, Virginia, p 7

Eternal Technology Corporation (2011) Material safety data sheet of formaldehyde. Eternal Technology Corporation, Virginia

Fernandes TF, Eleftheriou A, Ackefors H, Eleftheriou M, Ervik A, Sanchez M et al (2001) The scientific principles underlying the monitoring of the environmental impacts of aquaculture. J Appl Ichthyol 17(4):181–193

Fernández N (2002) Evaluación biológica de la contaminación marina costera mediante bioensayos con embriones del erizo de mar *Paracentrotus lividus*. Doctoral thesis, Universidad de Vigo, Vigo, Spain

Flaherty M, Szuster B, Miller P (2000) Low salinity inland shrimp farming in Thailand. Ambio 29(3):174–179

Gräslund S, Bengtsson BE (2001) Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment—a review. Sci Total Environ 280(1–3):93–131

Hall LW, Anderson RD (1995) The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. Crit Rev Toxicol 25(4):281–346

Halling-Sørensen B (2001) Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. Arch Environ Contam Toxicol 40(4):451–460

Halling-Sørensen B, Sengeløv G, Ingerslev F, Jensen LB (2003) Reduced antimicrobial potencies of oxytetracycline, tylosin, sulfadiazin, streptomycin, ciprofloxacin, and olaquindox due to environmental processes. Arch Environ Contam Toxicol 44(1): 7–16

Hernando MD, De Vettori S, Martínez Bueno MJ, Fernández-Alba AR (2007) Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere 68(4): 724–730

Heuer OE, Kruse H, Grave K, Collignon P, Karunasagar I, Angulo FJ (2009) Human health consequences of use of antimicrobial agents in aquaculture. Clin Infect Dis 49(8):1248–1253

Holten-Lützhøft CH, Halling-Sørensen B, Jørgensen SE (1999) Algal toxicity of antibacterial agents applied in Danish fish farming. Arch Environ Contam Toxicol 36(1):1–6

Huntington TC, Roberts H, Cousins N, Pitta V, Marchesi N, Sanmamed A et al (2006) Some aspects of the environmental impact of aquaculture in sensitive areas. Report to the DG fish and maritime affairs of the European Commission. Poseidon Aquatic Resource Management, Hampshire, p 305

Isidori M, Lavorgna M, Nardelli A, Pascarella L, Parrella A (2005) Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci Total Environ 346(1–3):87–98

Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) (1997) Towards safe and effective use of chemicals in coastal aquaculture. Reports and studies, vol 65. Food and Agriculture Organization, Rome, p 126

King CK, Riddle MJ (2001) Effects of metal contaminants on the development of the common Antarctic sea urchin *Sterechinus neumayeri* and comparisons of sensitivity with tropical and temperate echinoids. Mar Ecol Prog Ser 215:143–154

Kobayashi N (1977) Preliminary experiments with sea urchin pluteus and metamorphosis in marine pollution bioassay. Publ Seto Mar Biol Lab 24(1):9–21

Kobayashi N, Okamura H (2004) Effects of heavy metals on sea urchin embryo development. 1. Tracing the cause by the effects. Chemosphere 55(10):1403–1412

Kobayashi N, Tamato S, Harino H, Kitano M (2008) A bioassay using sea urchin egg development to identify organotin pollution in sea water. Coast Mar Sci 32(1):77–81

Körner S, Das SK, Veenstra S, Vermaat JE (2001) The effect of pH variation at the ammonium/ammonia equilibrium in wastewater and its toxicity to Lemna gibba. Aquat Bot 71(1):71–78

Kungolos A, Emmanouil C, Tsiridis V, Tsiropoulos N (2009) Evaluation of toxic and interactive toxic effects of three agrochemicals and copper using a battery of microbiotests. Sci Total Environ 407(16):4610–4615

Kyerematen GA, Ogunlana EO (1987) An integrated approach to the pharmacological evaluation of traditional materia medica. J Ethnopharmacol 20(3):191–207

Lalumera GM, Calamari D, Galli P, Castiglioni S, Crosa G, Fanelli R (2004) Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54(5):661–668

Lera S, Pellegrini D (2006) Evaluation of the fertilization capability of *Paracentrotus Lividus* sea urchin storaged gametes by the exposure to different aqueous matrices. Environ Monit Assess 119(1):1–13

Lera S, Macchia S, Pellegrini D (2006) Standardizing the methodology of sperm cell test with *Paracentrotus Lividus*. Environ Monit Assess 122(1):101–109

Littler MM, Murray SN (1975) Impact of sewage on the distribution, abundance and community structure of rocky intertidal macroorganisms. Mar Biol 30:277–291

Lorenzo JI, Nieto O, Beiras R (2002) Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquat Toxicol 58(1–2):27–41

Losso C, Novelli AA, Picone M, Marchetto D, Pantani C, Ghetti PF et al (2007) Potential role of sulfide and ammonia as confounding factors in elutriate toxicity bioassays with early life stages of sea urchins and bivalves. Ecotoxicol Environ Saf 66(2):252–257

Mankiewicz-Boczek J, Nalecz-Jawecki G, Drobniewska A, Kaza M, Sumorok B, Izydorczyk K et al (2008) Application of a microbiotests battery for complete toxicity assessment of rivers. Ecotoxicol Environ Saf 71(3):830–836

Marín-Guirao L, Atucha AM, Barba JL, López EM, Fernández AJG (2005) Effects of mining wastes on a seagrass ecosystem: metal accumulation and bioavailability, seagrass dynamics and associated community structure. Mar Environ Res 60(3):317–337

Matranga V, Zito F, Costa C, Bonaventura R, Giarrusso S, Celi F (2010) Embryonic development and skeletogenic gene

expression affected by X-rays in the Mediterranean sea urchin *Paracentrotus lividus*. Ecotoxicology 19(3):530–537
Moulin L, Catarino AI, Claessens T (1816) Dubois P (2011) Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816). Mar Pollut Bull 62(1):48–54
Muchmore D, Epel D (1973) The effects of chlorination of wastewater on fertilization in some marine invertebrates. Mar Biol 19(2):93–95
Muñoz I, Martínez Bueno MJ, Agüera A, Fernández-Alba AR (2010) Environmental and human health risk assessment of organic micro-pollutants occurring in a Spanish marine fish farm. Environ Pollut 158(5):1809–1816
National Research Council (1993) Nutrient requirements of fish. Nutrient requirements of domestic animals. National Academy Press, Washington DC, p 114
Nikaido H (2009) Multidrug resistance in bacteria. Annu Rev Biochem 78:46–119
Park S, Choi K (2008) Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17(6):526–538
Pouliquen H, Delépée R, Larhantec-Verdier M, Morvan M-L, Le Bris H (2007) Comparative hydrolysis and photolysis of four antibacterial agents (oxytetracycline, oxolinic acid, flumequine and florfenicol) in deionised water, freshwater and seawater under abiotic conditions. Aquaculture 262(1):23–28
Rey-Asensio A, Carballeira C, Viana IG, Carballeira A (2010) Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioacumulación de microcontaminantes. In: Rey-Méndez M. LC, Fernández Casal J, Guerra A (eds) Foro dos Recursos mariños e da Acuicultura das Rías galegas XIII. USC, O Grove, pp 201–218
Ricco G, Tomei MC, Ramadori R, Laera G (2004) Toxicity assessment of common xenobiotic compounds on municipal activated sludge: comparison between respirometry and Microtox®. Water Res 38(8):2103–2110
Ritz C, Streibig JC (2005) Bioassay analysis using R. J Stat Softw 12(5):1–22
Ritz C, Streibig JC (2012) Analysis of dose-response curves. Drc package, p 140. Available at: http://www.bioassay.dk/. Accessed 21 Feb 2012
Saco-Álvarez L, Durán I, Ignacio Lorenzo J, Beiras R (2010) Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicol Environ Saf 73(4):491–499
Sano LL, Mapili MA, Krueger A, Garcia E, Gossiaux D, Phillips K et al (2004) Comparative efficacy of potential chemical disinfectants for treating unballasted vessels. J Great Lakes Res 30(1):201–216
Sapkota A, Sapkota AR, Kucharski M, Burke J, McKenzie S, Walker P et al (2008) Aquaculture practices and potential human health risks: current knowledge and future priorities. Environ Int 34(8):1215–1226
R Development Core Team (2008) R: A language and environment for statistical computing. Vienna Austria R Foundation for Statistical Computing (1), p 7
Thomulka KW, McGee DJ, Lange JH (1993) Use of the bioluminescent bacterium *Photobacterium phosphoreum* to detect potentially biohazardous materials in water. Bull Environ Contam Toxicol 51(4):538–544
Tolls J (2001) Sorption of veterinary pharmaceuticals in soils: a review. Environ Sci Technol 35(17):3397–3406
United States Environmental Protection Agency (1994a) Short-term methods for estimating the chronic toxicity of effluents and receiving water to west coast marine and estuarine organisms, 3rd edn. USEPA, Cincinnati, p 370
United States Environmental Protection Agency (1994b) ERL-Duluth's aquatic ecotoxicology data systems. http://www.epa.gov/eerd/westmethman.htm. Accessed 14 Aug 2011
United States Environmental Protection Agency (1995) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms, 1st edn. Office of Research and Development, Cincinnati, p 673
Van der Grinten E, Pikkemaat MG, Van den Brandhof E-J, Stroomberg GJ, Kraak MHS (2010) Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphere 80(1):1–6
Van Wijk DJ, Kroon SGM, Garttener-Arends ICM (1998) Toxicity of chlorate and chlorite to selected species of algae, bacteria, and fungi. Ecotoxicol Environ Saf 40(3):206–211
Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64(4):655–671
Wollenberger L, Halling-Sørensen B, Kusk KO (2000) Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. Chemosphere 40(7):723–730
World Health Organization (1993) Biomarkers and risk assessment: concepts and principles. Environmental health criteria, vol 155. WHO, Geneva, p 82
Xu X, Li Y, Wang Y, Wang Y (2011) Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay. Toxicol In Vitro 25(1):294–300
Yuan F, Hu C, Hu X, Wei D, Chen Y, Qu J (2011) Photodegradation and toxicity changes of antibiotics in UV and UV/H_2O_2 process. J Hazard Mater 185(2–3):1256–1263

Assessing the toxicity of chemical compounds associated with marine land-based fish farms: The use of mini-scale microalgal toxicity tests

M.R. De Orte[1], C. Carballeira[1], I.G. Viana[2], A. Carballeira[3]

[1]Departamento de Química Física, Cátedra UNESCO/UNITWIN/WICOP, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro s/n, 11510 Puerto Real, manoela.romano@uca.es.
Cádiz, Spain.
[2]Instituto Español de Oceanografía, Centro Costero de A Coruña, 130, 15080, A Coruña, Spain.
[3]Ecotoxicología, Departamento de Ecología. Facultad de Biología. Universidad de Santiago de Compostela, 15782, Santiago de Compostela, A Coruña, Spain.

Abstract

Many chemicals that are currently used in aquaculture have not been evaluated with regard to their specific effects on the aquatic environment. In the present study, the toxic effects of several chemicals associated with land-based marine fish farming activities were assessed using two species of marine microalgae (*Phaeodactylum tricornutum* and *Isochrysis galbana*). Mini-scale toxicity tests were performed with six antibiotics (amoxicillin, ampicillin, flumequine, oxytetracycline, streptomycin and sulfadiazine) and two disinfectants (formaldehyde and hypochlorite). Amoxicillin and streptomycin did not exert toxic effects. Sulfadiazine was the most toxic chemical; the EC_{50} values were 0.11 mg/l and 1.44 mg/l for *P. tricornutum* and *I. galbana* respectively. As expected, the disinfectants displayed high toxicity, and *P. tricornutum* was particularly sensitive to these compounds.
Although the differences in microalgal sensitivity depended on the chemical considered, both species were highly sensitive to most of the compounds tested. We strongly recommend the inclusion of mini-scale microalgal toxicity tests in environmental risk assessment (ERA) and environmental monitoring plans because they are cost-effective and rapid.

Keywords: Bioassay; Ecotoxicity; Growth test; Microplate; Phytoplankton; Biomonitoring.

1. Introduction

Growth of the aquaculture industry has been accompanied by the use of chemicals originally developed for other industrial sectors [1]. Disinfectants

and medicines are the chemicals most often used (and in the greatest quantities) on marine land-based fish farms. Use of these chemicals is essential for manipulating fish reproduction, increasing feeding efficiency, improving survival and growth rates, cleaning facilities, controlling pathogens and diseases, and reducing transport stress [2-6].

Antibacterial agents are frequently administered to farmed fish in order to prevent or combat pathogens. In addition, formaldehyde and hypochlorite are commonly used as pesticides or to disinfect tanks [1]. All of these chemicals are subsequently released directly into the marine environment [7]. Although there are some regulations regarding the discharge of fish farm wastes, the technology used in the aquaculture industry has developed faster than studies on the associated environmental effects of the effluents [7]. In addition, concern about public perception of the environmental impacts of such activities has led to aquaculturists being quite secretive about the use of chemicals [8]. Therefore, little is known about the amounts of chemicals discharged and the subsequent effects on the surrounding environment [9], especially regarding their toxicity.

Since the 1970s great efforts have been made to develop methods of assessing ecological hazards and risks in order to evaluate new and existing chemicals used in aquatic environments [10]. However, researches on the impact of effluents from land-based marine fish farms have been given less attention [11]. In this context, toxicity data and identification of an ideal test species are essential to provide accurate environmental risk assessment [12].

Microalgae have been recommended as test organisms because of their ecological relevance [13], sensitivity [14] and also because they are usually the first organisms to be affected [15]. Algal growth inhibition bioassays have traditionally been conducted in standardized Erlenmeyer flasks and growth evaluated by direct cell counting [16, 17]. Bioassays have recently been miniaturized (small scale performance) so that they can be carried out in cuvettes, scintillation tubes and microplates [18]. Different research groups have compared tests carried out with standard Erlenmeyer flasks and with microplate assays, and have reported consistent results [14, 19]. These studies emphasized the advantages of microplate assays, as larger numbers of samples and replicates can be used in less space than required for

standard tests. Therefore, microplate bioassays with microalgae appear to be useful tools for environmental risk assessment.

The main objective of the present study was to verify differences in the sensitivity between two species of marine microalgae, *Phaeodactylum tricornutum* (Bacillariophyceae) and *Isochrysis galbana* (Haptophyceae) to chemicals commonly detected in aquaculture discharges. The influence of six antibiotics -amoxicillin (AMOX), ampicillin (AMP), flumequine (FLU), oxytetracycline (OTC), streptomycin (ST) and sulfadiazine (SFD) - and two disinfectants-formaldehyde (CH_2O) and hypochlorite (NaClO) – on the growth of the microalgae species were evaluated by miniaturized bioassay method.

2. Materials and methods

2.1. Test chemicals

The following test compounds (% purity) were purchased from Sigma Aldrich®: amoxicillin (>99.0), ampicillin (99), flumequine (>99.0), oxytetracycline (>95), streptomycin (>98.0), sulfadiazine (>99.0), formaldehyde (37%) and sodium hypochlorite (10%).

AMOX, AMP, OTC, ST, formaldehyde and hypochlorite were dissolved in sterilized seawater. FLU and SFD were dissolved in 0.05 M NaOH. The pH of each substance was adjusted to 8.0 ± 0.5 with HCl or NaOH (0.1 N), and the desired concentrations were obtained by dilution of the substances in sterilized seawater. The physicochemical parameters of the antibiotics tested are shown in Table 1.

Table 1. Physico-chemical properties of the chemicals tested (Holten-Lützhøft et al., 1999, Beausse 2004, Chee-Sanford et al., 2009).

Chemical	Water solubility (mg/l)	log Kow	Koc	Molecular weight	Half-life in water (25ºC) (days)	pKa
Amoxicillin	3433	0.87	865.5	365	$1.87E^{+16}$	2.4-9.6
Ampicillin	439.3	1.35	534.4	349	$1.91E^{+12}$	2.53-7.24
Flumequine	308.4	1.72-2.60	71.25	261	$4.57E^{+8}$	6.3
Formaldehyde	$5.70E^{+4}$	0.35	1	30	39.69	-4.2

Hypochlorite	$1.00E^{+6}$	-0.87	14.3	51	$1.38E^{+14}$	7.5
Oxytetracycline	313-1000	-0.9-(-1.22)	97.2	460	$3.08E^{+20}$	3.3-9.1
Streptomycin	20000	-7.53	10	582	54	4.5-8.8
Sulfadiazine	$2.81E^{+4}$	-0.09-0.12	188.9	250	$2.44E^{+05}$	3.27-6.4

2.2. Culture conditions

Phaeodactylum tricornutum has been proposed as standard organism for seawater toxicity test [20]. Due to its easy cultivation, it is one of the most used algal specie in marine bioassays [21]. *I. galbana* is also frequently used in toxicity test because it is recognized as sensitive specie [22]. A part from their ecotoxicological importance, both species were also chosen due to their wide distribution along the Galician coast (native organisms) [23] and also because they are frequently used in aquaculture as food for other organisms.

Phaeodactylum tricornutum (AROSA) and *Isochrysis aff. galbana* (Clon T-ISO) were obtained from the algal collection owned by the Department of Microbiology and Parasitology (University of Santiago de Compostela). Algae were pre-cultured in 100mL sterilized natural seawater enriched with *ALGAL* medium [23] in previously autoclaved Erlenmeyer flasks (250mL). The Erlenmeyer flasks were fitted with transpirable tops and maintained at 22□C under a photoperiod of 16 hours light/8 hours dark and irradiated at 160µ$Em^{-2}s^{-1}$. The cultures were shaken gently once a day, to ensure adequate aeration. The environmental conditions during the study were in accordance with international standards for such tests [17].

2.3. Toxicity tests

The medium used in the toxicity tests was the same as that used for pre-culturing the algae (*ALGAL* medium), but was diluted 6 times to allow at least 16 times growth of the algal population, while minimizing any interference by the medium in the toxicity values.

The chemicals were added to the diluted medium containing exponentially-growing algal cells (4-5 days old). The cells used in the tests were taken from the pre-cultures and initial cell density was established at 10^5 cell/ml (measured in a Neubauer chamber in an optical microscope). More than five concentrations (chosen from the results of preliminary tests) were tested

for each contaminant, and replicates were performed in quadruplicate for each concentration. The volume of test solutions was 300µL.

All tests were performed in 96-well flat-bottom microplates (Sero-Wel Bibby Sterelin Ltd, Stone, Staffs, UK). The microplates were maintained under the same conditions as the pre-cultures.

2.4. Growth inhibition

Growth of the algae was determined every 24h for 96h by measuring the absorbance of the growth medium (450nm) in a microplate reader (BECKMAN COULTER DTX 880 multimode detector). The inhibition of growth caused by each toxicant was determined by comparing the cell density measures with the cell density of the control medium (with sterilized seawater).

2.5. Statistical analysis

Growth curves of microalgae population were obtained by plotting absorbance values and developmental time. The areas under the growth curves were calculated and compared with the corresponding areas for the controls, and the inhibition percentages were calculated from these data, as described in OECD [16]. The effective concentration (EC_x) of a substance is defined as the concentration that causes a defined growth inhibition (x) of a microalgal population. The resulting data were used to perform a non-linear regression analysis, with R software and drc add-on package [24], to determine effective concentration (EC) values for the different chemicals used in the test [25]. No observed effect concentrations (NOEC), lowest observed effect concentration (LOEC), and significant differences between growth rates were determined by ANOVA, followed by Dunnett's test, by use of SPSS 17.0 statistical software. The differences were classified according to their statistical significance, as follows: a ($p < 0.001$), b ($p < 0.01$) and c ($p < 0.05$).

3. Results

3.1. Microalgal growth response

The dose-response curves obtained for antibiotics and disinfectants tested with *P. tricornutum* and *I. galbana* are shown in Figure 1 together with the statistical significant values. AMOX did not exert any effect on either species, and therefore this antibiotic is not included in the figure; the same applies to *I. galbana* and FLU. The EC50 is widely used and is regarded as the most robust end point in toxicological studies, but is not considered as a protective criterion [26]. The low toxicity and solubility of some antibiotics prevented tests being carried out with higher concentrations in many cases. Therefore, when possible, EC_5, EC_{10} and EC_{20} values were calculated in addition to NOEC and LOEC values. The ECs, NOEC and LOEC values are shown in Table 2 and 3.

3.1.1. Antibiotics

AMOX had no effect on either species, and the NOEC values (250mg/l) did not represent realistic environmental concentrations. AMP caused a slight (EC_{20} = 80.32mg/l) decrease in the density of *P. tricornutum*, while the same antibiotic slightly stimulated growth of *I. galbana*. FLU reduced development of *P. tricornutum* but did not have any significant effect on *I. galbana*. The EC_{20} values for FLU corresponded to the maximum concentration tested (30 mg/l) for *P. tricornutum,* whereas the same concentration did not have any effect on growth of *I. galbana*. OTC and ST inhibited growth of *I. galbana* in all treatments, and therefore the NOEC values were not able to be calculated. The same applies to AMP and *P. tricornutum*.

Among the antibiotics tested, SFD was the most toxic to both species of microalgae (with EC_{50} values of 0.11 and 1.44 mg/l for *P. tricornutum* and *I. galbana* respectively), followed by OTC. ST did not exert any effects on *P. tricornutum*, while growth of *I. galbana* was slightly stimulated (similar effects as AMP).

Table 2. EC values for the microalgal growth inhibition test performed with *P. tricornutum* and *I. galbana*.

	P. tricornutum				*I. galbana*			
Chemical (mg/l)	**EC_5**	**EC_{10}**	**EC_{20}**	**EC_{50}**	**EC_5**	**EC_{10}**	**EC_{20}**	**EC_{50}**
Antibiotics								
Amoxicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ampicillin	n.d.	n.d.	80.32(n.d.)	n.d.	n.d.	n.d.	n.d.	n.d.
Flumequine	5.11(±0.34)	5.46(±0.49)	6.10(±0.97)	n.d.	n.d.	n.d.	n.d.	n.d.
Oxytetracycline	0.19(±0.03)	0.33(±0.03)	0.61(±0.04)	1.73(±0.15)	n.d.	n.d.	n.d.	6.43(±10.01)
Streptomycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfadiazine	0.02(±0.01)	0.02(±0.01)	0.03(±0.008)	0.11(±0.04)	0.12(±0.08)	0.18(±0.10)	0.32(±0.10)	1.44(±1.09)
Disinfectants								
Formaldehyde	n.d.	n.d.	2.04(±1.49)	3.29(±1.79)	n.d.	n.d.	n.d.	3.05(±54.63)
Hypochlorite	n.d.	n.d.	n.d.	0.67(±6.26)	n.d.	n.d.	8.00(±48.77)	5.99(±33.37)

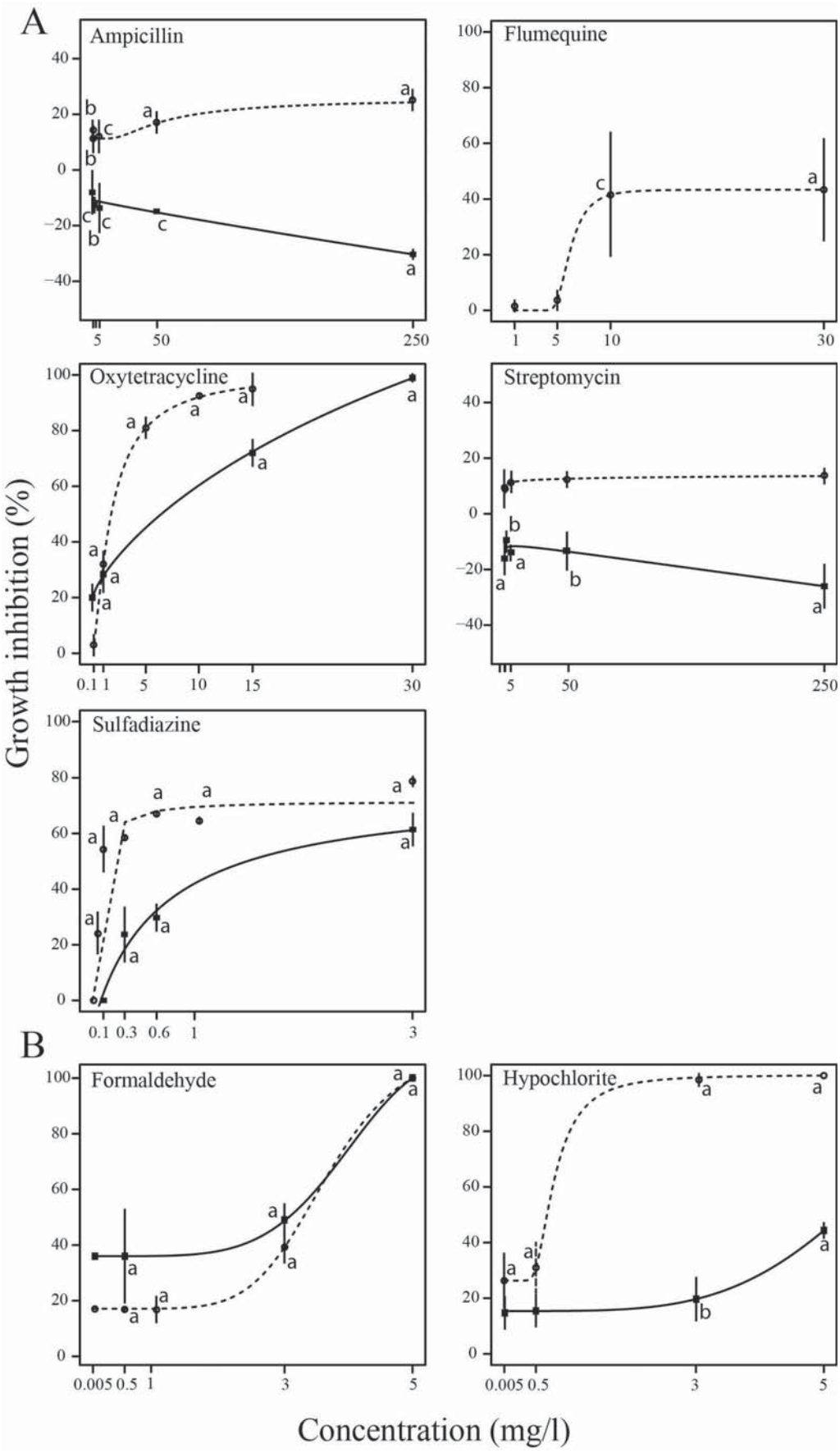


Figure 1. Dose-response curves of 5 antibiotics and 2 disinfectants tested with the microalgae *Isochrysis galbana* (solid line) and *Phaeodactylum tricornutum* (dashed line).

3.1.2. Disinfectants

Formaldehyde and hypochlorite strongly inhibited the growth of both species of microalgae, with LOEC (*I. galbana*) and NOEC (*P. tricornutum*) values of 0.005 mg/l.

Isochrysis galbana was slightly more sensitive to formaldehyde (EC_{50}= 3.05 mg/l) than *P. tricornutum* (3.29 mg/l), while the growth of the latter was affected by lower concentrations of hypochlorite (EC_{50}=0.67 mg/l).

Table 3. NOEC and LOEC values from the microalgal growth inhibition test performed with *P. tricornutum* and *I. galbana* with different antibiotics and disinfectants.

	P. tricornutum		*I. galbana*	
Chemical	**NOEC**	**LOEC**	**NOEC**	**LOEC**
Antibiotics (mg/l)				
Amoxicillin	250	n.d.	250	n.d.
Ampicillin	n.d.	0.05	n.d.	0.05
Flumequine	5	10	30	n.d.
Oxytetracycline	0.1	1	n.d.	0.1
Streptomycin	250	n.d.	n.d.	0.05
Sulfadiazine	0.01	0.05	0.1	0.3
Disinfectants (mg/l)				
Formaldehyde	n.d.	0.5	0.005	0.5
Hypochlorite	n.d.	0.005	0.5	3

3.2. Differences in microalgal sensitivity

The dose-response curves revealed that the difference in the sensitivity of the species depended on the antibiotic, although *P. tricornutum* appeared to be more sensitive considering the values of toxic parameters. OTC and SFD were the only antibiotics that enabled calculations of EC50 values for both species and the values for *P. tricornutum* were always lower (higher toxicity). The LOEC values confirmed a higher sensitivity for *P.tricornutum*. When calculation of LOEC was possible for both of the species, *P. tricornutum* presented lower values in most of the cases.

There were no significant differences between formaldehyde EC values for either microorganism. *Phaeodactylum tricornutum* was approximately one order of magnitude more sensitive to hypochlorite than *I. galbana*, although the results of the *I. galbana* toxicity assay were highly variable and less reliable than those of the P. *tricornutum* assay.

4. Discussion

In comparison with cage systems, land-based fish farming is not considered to have a great impact on the environment [27]. However, fish farm effluents containing large amounts of chemicals and organic wastes are often discharged directly into coastal habitats [26], and may have important effects on the ecosystem.

4.1. Antibiotics

AMOX was the only product that did not cause any interference in the growth of either species, as observed in previous studies. Holten-Lützhøft et al. [28] evaluated the toxicity of AMOX with the cyanobacterium *Microcystis aeruginosa*, the freshwater green algae *Selenastrum capricornutum* and the marine cryptophycean *Rhodomas salina*, and found that only the cyanobacterium was sensitive to the compound. Surprisingly, AMOX did not display any toxic effects in a 30 min standard bioluminescence inhibition assay with *Vibrio fisheri* [29], even though AMOX is an antibiotic.

AMP has not been reported to display high toxicity in comparison with other medicines. Eguchi et al. [30] reported EC50 values above 1000 mg/l for AMP and the freshwater green algae *Selenastrum capricornutum* and *Chlorella vulgaris* in 72 hours growth inhibition tests. Carballeira et al. [31] observed that a concentration of AMP higher than 100 mg/l improved development of sea urchin larvae; they also found the same response for ST. These authors emphasized that AMP and ST may be used in aquaculture as growth stimulators rather than for disease prevention. In the present study, microalgae responded differently to these products; development of *I. galbana* was stimulated by both products while growth of *P. tricornutum* was slightly inhibited by AMP, and ST did not produce any response. Both antibiotics are widely used on land-based fish farms, because of their

relatively low toxicity to fish, although information on the exact quantities used is not available [4, 5], as these data are only published in confidential technical reports [9].

Growth inhibition assays (7 days) carried out with microalgae revealed EC_{50} FLU values of 5 mg/l for *S. capricornutum* and 18 mg/l for *R. salina* [28]. Both of the microalgae species tested in the present study were less sensitive to this compound. On the other hand, both species showed higher sensitivity to SFD (with EC50 values below 0.15 mg/l) than in previous toxicity studies [28]. These findings indicate that toxic responses will differ significantly between species, depending on the product. It is therefore recommended that several species of microalgae are included in test batteries of chemicals since there is no single most sensitive species [26].

The EC50 values for OTC (1.73 and 6.43 mg/l for *P. tricornutum* and *I. galbana* respectively) are within the range of values reported in the literature for other microalgal species [28, 30, 32]. These results suggest that microalgae are among the most sensitive organisms to OTC, with lower EC_{50} values than fish, bacteria and most invertebrates [29, 32-34]. Microalgae have generally been found to be more sensitive than other organisms in tests with pharmaceuticals [33, 35]. However, since such chemicals are generally designed to fight bacteria, cyanobacteria were particularly sensitive. Toxicity tests with *M. aeruginosa* have revealed that this species was two to three orders of magnitude more sensitive to AMOX, FLU, OTC, SFD and other antibiotics than both *S. capricornutum* and *R. salina* [28]. These results were confirmed by Halling-Sørensen [35] with eight antibiotics (including ST) and the same species. Therefore cyanobacteria appear to be the best candidates for assessing the toxicity of these pharmaceuticals.

Antibiotics are usually present at low concentrations in the environment. Nevertheless, they are widely used in aquaculture, and therefore may be present almost continuously in some ecosystems, leading to chronic exposures of wild organisms [32]. Most of the antibiotics tested have a long half-life when dissolved in water (Table 1), and therefore low concentrations combined with long term effects (chronic toxicity) should be considered [33]. Changes in exposure time from 5 to 7 days may decrease EC_{50} values by one order of magnitude [36]. Therefore, the duration of microalgal test

(96 h) may reflect chronic toxicity. Furthermore, these substances may interact with each other causing synergistic effects, and the toxicity of antibiotics may be enhanced [30], while negative effects on microalgae may extend to higher trophic levels and affect the whole ecosystem.

Antibiotics have shown selective toxicity. Microalgae are particularly sensitive to medicines, and are sometimes the most sensitive organisms [30, 32, 33].

4.2. Disinfectants

Regulations for maximum permissible levels of disinfection by-products have been established by the World Health Organization (WHO). The value established for formaldehyde was 0.9mg/l [37]. Considering the lowest EC value obtained in the present study was 2.04 (EC_{20}) for *P. tricornutum*, WHO regulations were satisfactorily conservative.

In order to assess the environmental risk associated with formaldehyde, Chénier [38] compiled a large data set from toxicity studies with algae, microorganisms, invertebrates, fish and amphibians. The most sensitive toxic effect identified was for the brown marine macroalgae *Phyllospora comosa*, with concentrations of 0.1 mg/l causing 40% mortality in 96 hour experiments with day-old zygotes. While values for other organisms were generally higher than those found in this study for both species of microalgae tested, there are no data from microalgal toxicity tests. The EC_{50} values obtained in this study for *P. tricornutum* (3.29mg/l) were only slightly higher than those found for *I. galbana* (3.05mg/l). However, for the latter species, the results of the toxicity assay were very variable, and consequently the test is less replicable and reliable. The low replicability may occur because *I. galbana* occasionally forms aggregates [39], and therefore the readings may correspond to the aggregates in some samples, but not in others. The same applies to hypochlorite. Thus, for some compounds, measuring absorbance values in a microplate reader may not be the most appropriate method for detecting toxicity to *I. galbana*. It has been suggested that for filamentous, aggregating and autospore-forming algal species, measurement of chlorophyll fluorescence may be the best method of estimating microalgal biomass in a microplate reader [15].

Hypochlorite is one of the most commonly used disinfectants worldwide because of its low cost and high effectiveness. In solution, it reacts quickly, generating a variety of organic chlorinated compounds that are toxic in aquatic environments [40]. *Phaeodactylum tricornutum* was found to be highly sensitive to hypochlorite (EC_{50} 0.67mg/l). López-Galindo et al [41] reported an EC_{50} of 1.73mg/l for *Dunaniella salina* and 2.91mg/l for *I. galbana*. Nevertheless, it is recognised that microalgae are less sensitive than other organisms to this compound [40].

Chlorate is a by-product of hypochlorite and typically accounts for less than 1% of commonly used disinfectants [42]. However, chlorate is mainly considered to be toxic to marine algae and some microorganisms [43]. Chlorine, also contained in hypochlorite, is acutely toxic to marine organisms, with EC_{50} values equal or less than 1mg/l [40].

The different toxicity values for the contaminants tested with microalgal species show that microalgal sensitivity is chemical-specific. Furthermore, microalgae indicated the toxicity of most of these chemicals at lower concentrations than indicated by other organisms. Therefore microalgae should be included in environmental risk assessment studies, particularly the marine microalgae *P. tricornutum* because of the high replicability of the results obtained. However, the use of a battery of test species from different trophic levels is recommended for accurate risk assessment [44].

Little is known about the biological responses to different concentrations of chemicals used in aquaculture [26]. The data obtained in this study are useful for estimating the potential environmental hazards associated with land-based marine aquaculture activities.

The use of mini-scale toxicity tests enables performance of bioassays with a large number of chemicals and replicates in a sensitive, rapid and cost-effective manner. Therefore, the application of such tests is also recommended for environmental monitoring. Nevertheless, microalgae displayed selective sensitivity, so that diverse species and different organisms must be considered to cover a wide spectrum of toxicity.

Acknowledgments

This work was funded by the National Plan for Marine Culture (JACUMAR 2008) within a project entitled "*Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos y medidas para estudios ambientales en acuicultura marina* (INDAQUA)." M.R. De Orte, is grateful to the European Union for the Erasmus Mundus Scholarship provided. The authors are thankful to Dr. Jaime Fábregas of the Department of Microbiology and Parasitology (USC) for providing the microalgae and culture medium.

References

[1] GESAMP, *Towards safe and effective use of chemicals in coastal aquaculture. Reports and studies* nº 65 (1997).

[2] T.F. Fernandes, A. Eleftheriou, H. Ackefors, M. Eleftheriou, A. Ervik, M.A. Sanchez, T. Scanlon, P. White, S. Cochrane, T.H. Pearson and P.A. Read, *The scientific principles underlying the monitoring of the environmental impacts of aquaculture,* J. Appl. Ichthyol. 17 (2001), pp.181-193.

[3] T.C. Huntington, H. Roberts, N. Cousins, V. Pitta, N. Marchesi, A. Sanmamed, T. Hunter-Rowe, T.F. Fernandes, P. Tett, J. McCue and N. Brockie, N., 2006. *Some aspects of the environmental impact of aquaculture in sensitive areas. Report to the DG Fish and Maritime Affairs of the European Commission.* Poseidon Aquatic Resource Management, Hampshire, pp 305.

[4] A. Sapkota, A.R. Sapkota, M. Kucharski, J. Burke, S. McKenzie, P. Walker and R. Lawrence, *Aquaculture practices and potential human health risks: Current knowledge and future priorities,* Environ. Int. 34 (2008), pp. 1215-1226.

[5] L. Burridge, J.S. Weis, F. Cabello, J. Pizarro, and K. Bostick, *Chemical use in salmon aquaculture: A review of current practices and possible environmental effects*,

Aquaculture 306 (2010), pp. 7-23.

[6] Q. Zheng, R. Zhang, Y. Wang, X. Pan, J. Tang, and G. Zhang, G., *Occurrence and distribution of antibiotics in the Beubu Gulf, China: Impacts of river discharge and aquaculture activies*, Mar. Environ. Res. 78 (2012), pp. 26-33.

[7] C. Carballeira, J. Ramos-Gómez, M.L. Martín-Díaz, T.A. DelValls and A. Carballeira, *Designing an Integrated Environmental Monitoring Plan for Land-Based Marine Fish Farms Located at Exposed and Hard Bottom Coastal Areas*, J. Environ. Monitor. 14 (2012), pp. 1305-1316.

[8] V. Zitko, *Analytical chemistry in monitoring the effects of aquaculture: one laboratory´s perspective*, ICES J. Mar. Sci. 58 (2001), pp. 486-491.

[9] M. Crane, G.A. Burton, J.M. Culp, M.S. Greenberg, K.R. Munkittrick, R. Ribeiro, M.H. Salazar and S.D. St-Jean, *Review of aquatic in situ approaches for stressor and effect diagnosis*, Integr. Environ. Assess. Manag. 3 (2007), pp. 234-245.

[10] H. Selck, B. Riemann, K. Christoffersen, V.E. Forbes, K. Gustavson, B.W. Hansen, J.A. Jacobsen, O.K. Kush and S. Petersen, *Comparing sensitivity and ecotoxicological effect endpoints between laboratory and* field, Ecotox. Environ. Safe. 52 (2002), pp. 97-112.

[11] S. Vizzini and A. Mazzola, *Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean*, Mar. Pollut. Bull. 49 (2004), pp. 61-70.

[12] P. Pandard, J. Devillers, A.M. Charissou, V. Poulsen, M.J. Jourdain, J.F. Ferard, C. Grand and A. Bispo, *Selecting a battery of bioassays for ecotoxicological characterization of wastes*, Sci. Total. Environ. 363 (2006), pp. 114-125.

[13] P. Arensberg, V.H. Hemmingsen and N. Nyholm, *A miniscale algal toxicity test,* Chemosphere 30 (1995), pp. 2103-2115.

[14] Ž. Pavlić, B. Stjepanović, J. Horvatić, V. Peršić, D. Puntarić and J. Čulig, *Comparative sensitivity of green algae to herbicide using erlenmeyer flask and microplate growth inhibition assays*, B. Environ. Contam. Tox. 76 (2006), pp. 883-890.

[15] A. Satoh, L.Q. Vudikaria, N. Kurano and S. Miyachi, *Evaluation of the sensitivity of marine microalgal strains to the heavy metals, Cu, As, Sb, Pb and Cd*, Environ Int. 31 (2005), pp. 713-722.

[16] OECD, 2011. Guidelines for the testing of chemicals. Section 2: Effects on biotic systems test N° 201: Freshwater alga and cyanobacteria, growth inhibition test. Organisation for Economic Co-operation and Development, Paris, France.

[17] ISO (International Standard Organization), 1995. ISO 10253:1995(E). Water quality-marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*, pp 12.

[18] A. Eisentraeger, D. Dott, J. Klein and S. Hahn, *Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays*, Ecotox. Environ. Safe. 54 (2003), pp. 346-354.

[19] S.M. Paixão, L. Silva, A. Fernández, K. O'Rouke, E. Mendonça and A. Picado, *Performance of a miniaturized algal bioassay in phytotoxicity screening*, Ecotoxicology 17 (2008), pp. 165-171.

[20] I. Moreno-Garrido, L.M. Lubian and A.M.V.M. Soares, *Influence of cellular density on determination of EC_{50} in microalgal growth inhibition tests*, Ecotox. Environ. Safe. 47 (2000), pp. 112-116.

[21] J. Horvatić and V. Peršić, *The effect of Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} on the growth rate of marine diatom Phaeodactylum tricornutum Bohlin: Microplate growth inhibition test*, B. Environ. Contam. Tox. 79 (2007), pp. 494-498.

[22] P. Pérez, E. Fernández and R. Beiras, *Fuel toxicity on Isochrysis galbana and a coastal phytoplankton assemblage: Growth rate vs. variable fluorescence*, Ecotox. Environ. Safe. 73 (2010), pp. 254-261.

[23] J Fabregas, J. Abalde, C. Herrero, B. Cabezas and M. Veiga, *Growth of the marine microalga Tetraselmis suecica in batch cultures with different salinities and nutrient concentrations.* Aquaculture 42 (1984), pp. 207-215.

[24] C. Ritz and J.C. Streibig, *Bioassay analysis using R,* J. Stat. Softw. 12 (2005), pp. 1-22.

[25] R Development Core Team, 2008. R: A language and environment for statistica computing. R foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.

[26] C. Carballeira, M.R. De Orte, I.G. Viana, and A. Carballeira, A., *Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms.* Ecotox. Environ. Safe. 78 (2011), pp. 148-161.

[27] S. Vizzini and A. Mazzola, *Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean*, Mar. Pollut. Bull. 49 (2004), pp. 61-70.
[28] H.C. Holten-Lützhøft, B. Halling-Sørensen and S.E. Jørgensen, *Algal toxicity of antibacterial agents applied in Danish fish farming.* Arch. Environ. Con. Tox. 36 (1999), pp. 1-6.
[29] G.M. Lalumera, D. Calamari, P. Galli, S. Castiglioni, G. Crosa and R. Fanelli, *Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy.* Chemosphere 54 (2004), pp. 661-668.
[30] K. Eguchi, H. Nagase, M. Ozawa, Y.S. Endoh, K. Goto, K. Hirata, K. Miyamoto and H. Yoshimura, *Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae*, Chemosphere 57 (2004), pp. 1733–1738.
[31] C. Carballeira, M.R. De Orte, I.G. Viana, T.A. Del Valls and A. Carballeira, *Assessing the toxicity of Chemicals compounds associated with land-based marine fish-farms: the sea-urching embryo bioassay with Paracentrotus lividus and Arbacia lixula*, Arch. Environ. Con. Tox. 63 (2012), pp. 249-261.
[32] C.S.G. Ferreira, B.A. Nunes, J.M.M. Henriques-Almeida and L. Guilhermino, *Acute toxicity of oxytetracycline and florfenicol to the microalgae Tetraselmis chuii and to the crustacean Artemia parthenogenica*, Ecotox. Environ. Safe. 67 (2007), pp. 452-458.
[33] M. Isidori, M. Lavorgna, A. Nardelli, L. Pascarella and A. Parrella, *Toxic and genotoxic evaluation of six antibiotics on non-target organisms*, Sci. Total. Environ. 346 (2005), pp. 87-98.
[34] S. Park and K. Choi, *Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems.* Ecotoxicology 17 (2008), pp. 526-538.
[35] B. Halling-Sørensen, *Algal toxicity of antibacterial agents used in intensive farming.* Chemosphere 40 (2000), pp. 731-739.
[36] E. Van der Grinten, M.G., Pikkemaat, E. Van den Brandhof, G.J. Stroomberg and M.H.S. Kraak, *Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics*, Chemosphere 80 (2010), pp. 1-6.
[37] WHO, 1993. Biomarkers and risk assessment: concepts and principles. Environmental health criteria Vol 155, World Health Organization, Geneva.
[38] R. Chénier, *An ecological risk assessment of formaldehyde*, Hum. Ecol. Risk. Assess. 9 (2003), pp. 483-509.
[39] E.P. Espinosa and B. Allam, *Comparative growth and survival of juvenile hard clams, Mercenaria mercenaria, fed commercially available diets*, Zoo. Biol. 25 (2006), pp. 513-525.
[40] E. Emmanuel, G. Keck, J. Blanchar, P. Vermande, and Y. Perrodin, *Toxicological effects of disinfections using sodium Hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater.* Environ. Int. 30 (2004), pp. 891-900.
[41] C. López-Galindo, M.C. Garrido, J.F. Casanueva and E. Nebot, *Degradation models and ecotoxicity in marine waters of two antifouling compounds: Sodium hypochlorite and an alkylamine surfactant,* Sci. Total. Environ. 408 (2010), pp. 1779-1785.
[42] D.J. VanWijk and T.H. Hutchinson, *The ecotoxicity of chlorate to aquatic organisms: A critical review*, Ecotox. Environ. Safe. 32 (1995), pp. 244-253.

[43] D.J. VanWijk, S.G.M. Kroon and I.C.M. Garttener-Arends, *Toxicity of chlorate and chorite to selected species o algae, bacteria and fungi*, Ecotox. Environ. Safe. 40 (1998), pp. 206-2011.

[44] J. Mankiewicz-Boczek, G. Nalecz-Jawecki, A. Drobniewska, M. Kaza, B. Sumorok, K. Izydorczyk, M. Zalewski and J. Sawicki, *Application of a microbiotests battery for complete toxicity assessment of rivers*, Ecotox. Environ. Safe. 71 (2008), pp. 830-836.

Toxicity evaluation of commonly used biocides in land based marine fish farms using the miniaturized bioluminescence test with *Vibrio fischeri*

I. G.Viana[1], C. Carballeira[2], M. De Orte[2] & A. Carballeira[3]

[1]*Instituto Español de Oceanografía, Centro Costero de A Coruña, Apdo. 130, E-15080, A Coruña, Spain.*
[2]*UNITWIN/UNESCO/WiCoP. Departamento de Química-Física, CASEM, University of Cádiz,11510, Puerto Real, Spain.*
[3]*Ecología, Facultad de Biolgía, Universidad de Santiago de compostela, 15782, Santiago de Compostela, Spain.*

Abstract

Toxicity assessment of marine fish farm effluents (chronic and low concentration of contaminants) requires sensitive and rapid tools because of the recent growth of aquaculture activities. The miniaturized bioluminescence test with *V. Fischeri* was used to evaluate the toxicity of common chemicals (antibiotics, disinfectants and ammonia) from intensive land-based pisciculture. Different salinities and exposure times were assayed in order to adapt this test for the proper assessment of marine pisciculture effluents. Antibiotics (amoxicillin, ampicillin, flumequine, oxytetracycline, sulphadiazine and streptomycin) seemed to have a delayed toxicity, therefore, higher exposure times should be developed with the bioluminiscence test. On the contrary, disinfectants and ammonia were not affected by the exposure time. Ammonia, the main waste from fish metabolism, showed low toxicity, nevertheless, the unstability of this compound in solution leads to high standard deviation values. Increasing salinity greatly decreased antibiotics toxicity. Moreover, toxicity endpoints from miniaturized test were within the range of literature values but are generally smaller than those obtained from conventional methods.

Keywords: antibiotics, disinfectants, ammonium chloride, salinity, microplates,

1. Introduction

In recent years coastal environments has been subjected to an increased environmental pressure from human activities. Among these activities, aquaculture has received particular attention due to its increasing development and the potential for loading water and discharging effluents with polluting agents (Shahidul Islam and Tanaka, 2004). Aquaculture continues to grow more rapidly (8.8% per year) than all other animal food-producing sectors (FAO, 2006). Although there are some

regulations regarding the discharge of Land-Based Marine Fish Farms (LBMFFs) efluents, farming has developed faster than the knowledge of associated environmental effects (Carballeira et al., 2012). A wide range of chemicals are currently used in the aquaculture industry; disinfectants, antifoulants and veterinary medicines,... (Douet et al., 2009) which are essential to increase and control production of seed in hatcheries, increase feeding efficiency, improve survival rates, control pathogens and diseases and reduce transport stress (Huntington et al., 2006).

A serious environmental impact of land-based aquaculture occurs when untreated effluent is discharged into surroundings bodies of water (Cripps and Kumar, 2003). In this manner, the environmental impact is primarily a function of feed composition and feed conversion and pesticides (Tacon et al., 1995).

Chemical analysis was traditionally used to assess the environmental risk of contaminants generated by aquaculture industry (Muñoz et al., 2010). The high dilution, variability and the complex interactions of pollutants require expensive measures in order to have reliable analysis (Pouliquen et al., 2007, Rey-Asensio et al., 2010). Besides, this method has limitations when evaluating the bioavailability of contaminants (Hernando et al., 2006) either the effect of interaction among toxic substances (Wolska et al., 2007). In this way, the lack of predicting models make the approach based only on chemical analysis does not satisfy the requirements for an environmental monitoring plan.

In this sense, marine bioassays are becoming popular tools to assess the potential toxic risk of common and emerging chemicals (Hernando et al., 2006) with a quick, easy, cheap and ecologically relevant method (Beiras et al 2011).

Some authors have studied the toxicity of some chemicals applied in these aquaculture facilities (de Orte et al., 2010; Hernando et al., 2007; Holten-Lützhøft et al., 1999; Lalumera et al., 2004). However, their effects are not yet well documented.

Many studies have tried to develop rapid and sensitive bioassays to monitor and assess the toxic materials. Among toxicity tests, the bioluminescent inhibition test using the marine bacteria *Vibrio fischeri* has been widely applied in water and sediment analysis, isolated or within a battery of bioassays (Blaise 2000; Peters et al 2002; Carballeira et al., 2012). Microtox analyzers use *V. fischeri*, is one of the first commercialized microbiotest (Bulich, 1979) and it is also standardized in several

countries. Furthermore, it was found to be one of the most sensitive test (Parvez et al., 2006).

Over the last years, biological tests have been miniaturized (Blaise et al., 1994; Koutny and Zaoralkova, 2005; Paixao et al., 2008; Pavlic et al., 2006) to substitute laborious procedures by less expensive and more convenient methods (Hirmann et al., 2007). Among these studies, several authors have miniaturized the bioluminescence test with *V. fischeri* (Blaise et al., 1994; Fiehn et al., 1997; Froehner et al., 2002; Hirmann et al., 2007; Schmitz et al., 1999) and other bacterial species (Gellert and Stommel, 1999; Maxam et al., 2002; Reemtsma et al., 1999; Schmitz et al., 1998; Tamminen and Virta, 2007). Although the number of chemicals that have been tested are still scarce.

Bioassays are monitoring tools which try to estimate the likelihood that contaminants within an environmental matrix may cause effects at different levels of organization. The effects of biocides from LBMFFs include the altering of decomposer community, an essential component of ecosystems because of their role in the biogeochemical cycles of the elements... This effects may be different depending on the salinity of the bioassay or receiving environment because it alters the bioavailability/toxicity of contaminants.

Moreover, although the bioluminiscent test is already standardized some studies found more sensitive results when increasing the length of the test (Backhaus et al., 1997).

For this reasons, the aim of this work is to determine the toxicity of medicines, disinfectants and ammonium (fish metabolism) from LBMFFs with the miniaturized bioluminescence test with *V. ficheri* while testing the toxicity kinetics (15, 30 y 60 min) and the effect of salinity (20‰ and 36‰). In this manner, the method will be tunned for the risk assessment of effluents from intensive land-based marine fish farms and the toxicity values of commonly pollutants from these farms will be obtained.

2. Material and methods

2.1. Contaminants

The following chemicals (% purity) were selected: a metabolite, ammonium chloride (>99.5%); the antibiotics: amoxicillin, flumequine, oxytetracycline hydrochloride (95%), ampicillin, streptomycin sulphate and sulphadiazine (>99%); and two disinfectants: formaldehyde stabilized with methanol (35-40%) and sodium hypochlorite (10%).

Chemicals were purchased from Sigma Aldrich except formaldehyde and sodium hypochlorite which were purchased from Panreac Quimica.

Stock solutions of the chemicals were prepared with two different aqueous solutions (Mili Q pore water): 20‰ solution –as is standarized in the ISO 11348-3 (2007) protocol- and artifical 36‰ seawater solution (Zaroogian, 1969). The salinity of the final test solution of seawater was 28‰ because of the reconstitution of bacteria with the solution of 20‰. NaOH 0.05 M was used as solvent for sulphadiazine, amoxicillin, flumequine, ampicillin and streptomycin. Sulphadiazine solution was previously heated (35ºC) to ensure the complete solution of the antibiotic. Th pH of every solution was set to 7±1 with 0.1 N NaOH or HCl before conducting the toxicity tests in order to discard pH as confounding factor. From 7 to 15 different concentrations, obtained by adding the relevant sterilized solution, were tested with each contaminant. Special emphasis was given to the lower effects region. Stock solutions were done 12h before performing the tests in order to ensure the stability of chemicals solutions. Nominal concentrations were checked by tandem mass spectrometry (MS/MS) coupled to a HPLC. Solutions of NaOH 10 mM and acetonitrile/water (50:50) were previously prepared for the moving phases. Water and acetonitrile included 0.2 and 0.1% formic acid, respectively. Reference material and seawater were used for stock solutions, posteriorly filtered (0,2µm) and inyected in the HPLC-MSMS equipped with a Zorba Eclipse Plus column, 2.1 x 30 mm C18, 1.8.

2.2. *Vibrio fischeri* bioluminiscence test

Toxicity testing was evaluated using the bioassay based on the inhibition of the luminescence released by the marine bacteria *Vibrio fischeri* (strain NRRL-B-11177). For this purpose, Biotox commercial test kit (Azur Environmental) was used. Bacterial reagents were reconstituted just prior to the analysis, and the pre-incubations times and temperatures followed device protocols. Bacterial suspension and prepared dilutions were maintained at 15º C in a thermoblock while conducting the test.

Sterile 96 black well Microtiter® plates with clear bottom (ThermoFisher Scientific OY, Finland) were used (Hirmann et al., 2007). The bacterial suspension was added to the wells where each of them contained 100 µL of bacteria and 100 µL of chemical dilutions. Each sample was tested in triplicate.

Seven reference growth samples were included on the test and all samples were corrected by the mean value of blanks (minimum 8 blanks per microplate). The plates were covered with standard lids during the test to avoid losses due to the volatility. Phenol was used as reference standard to ensure validity of each vial and microplate tests. Replicas were considered valid only if they had a coefficient of variation lower than 15%.

Bioluminescence was measured using a DTX 880 Multidetector at 15, 30 and 60 minutes of incubation. Microplates were orbitally shaken during 5s prior to each measure (integration time of 1000ms).

The percentage of inhibition (INH%) was determined by comparing the growth response of chemical samples with that obtained with the reference. The decrease in bacterial luminescence (INH%) was determined for each sample through the following equation:

$$INH\% = \frac{Ic - It}{Ic} 100$$

Where Ic and It are the luminescence, at the same time of exposure (x), of reference and test sample respectively.

2.3. Statistical analysis

"Drc" package was used for the calculation of the concentration-response curves (Ritz and Streiberg, 2010) under R (R Development Core Team, 2009). For each compound the most suitable model of a pool of different parametric models was selected by their comparison using the maximum log likelihood value, Akaike´s information criterion (AIC), the estimated residual variance and the p-value form a lack-of-fit test as criteria. Toxic effects were expressed as effective concentrations (EC_{80}, EC_{50}, EC_{20}, EC_{10} and EC_5) when possible. Confidence intervals were calculated using asymptotics-based confidence intervals (using delta method and t-distribution).

The NOECs and LOECs values were determined for each chemical by application of a post hoc Dunnett test (ANOVA) under SPSS software (version 18.0).

3. Results

The internal quality control data for phenol are consistent with the recommended EC_{50} value range of 13.00–26.00 mg L^{-1} (Azur Environmental, 1998), indicating an

adequate physiological state of the tested microorganisms, according to the *V. fischeri* protocol. The small standard errors associated with EC_{50} values and the consistent data between assays indicate good accuracy of measures.

Figures 1 and 2 show the dose-reponse curves for each substance with the different salinities and performing times.

Toxicity determination (EC_5, EC_{10}, EC_{50}, EC_{80}, NOEC and LOEC) was calculated for 15, 30 and 60 min performing times, however, 30 min measures were a halfway response between the other times (Tables 1, 2 and 3). Only significant EC values and best fitting models are shown (Tables 1, 2 and 3). Solubility in seawater was the limiting factor of some of the antibiotics, therefore, some EC values could not be calculated (Fig. 1 and 2). Moreover, some EC values were estimated through the parametric model, those with high standard deviation were removed.

Only ammonium chloride and sodium hypochlorite (Table 1, Figure 1) showed a decrease of toxicity when testing with seawater compared to those made with 20 ‰ NaCl.

Toxicity depends on the salinity and the time that bacteria are exposed. In general, a higher exposure time is translated into higher toxicity values of antibiotics (Fig. 1 and 2). However, there were no clear differences for ammonium chloride and sodium hypochlorite.

The most toxic substance was formaldehyde, while the less toxic was ammonium chloride together with some antibiotics, such as sulphadiazine (Table 1, 2 and 3).

4. Discussion

4.1. Toxicity assessment of chemicals with the miniaturized test

There are various studies of miniaturization of luminescence test but this was the first time to test the selected substances. Microplate test procedure showed slightly lower light inhibition values (EC) than traditional cuvettes (Faust et al., 2001; Froehner et al., 2002; Parvez et al., 2008; Ren and Frymier, 2002; Ricco et al., 2004; Vaajasaari et al., 2004; Vighi et al., 2009) because of the different surface-volume relationship (Pavlic et al., 2006). However, our data match the results obtained with the traditional cuvettes confirming the validity of the miniaturized method (Isidori et al., 2005; Lalumera et al., 2004; Park and Choi, 2008; Pavlic et al., 2006; Ricco et al., 2004).

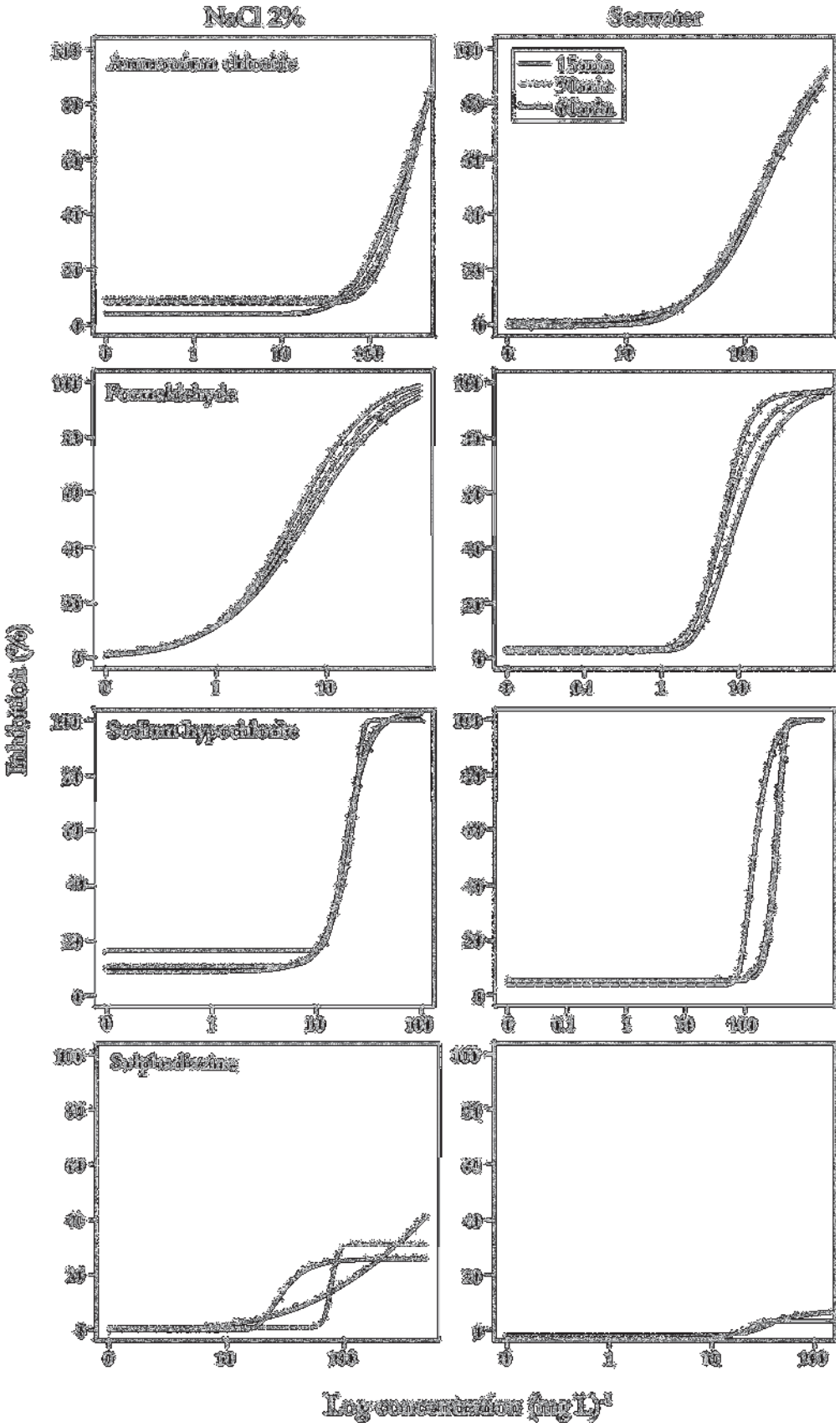


Figure 1. Concentration-response curves for the antibiotic sulphadiazine, the metabolite ammonium chloride and two disinfectants; formaldehyde and sodium hypochlorite. Different salinities and performing times are represented.

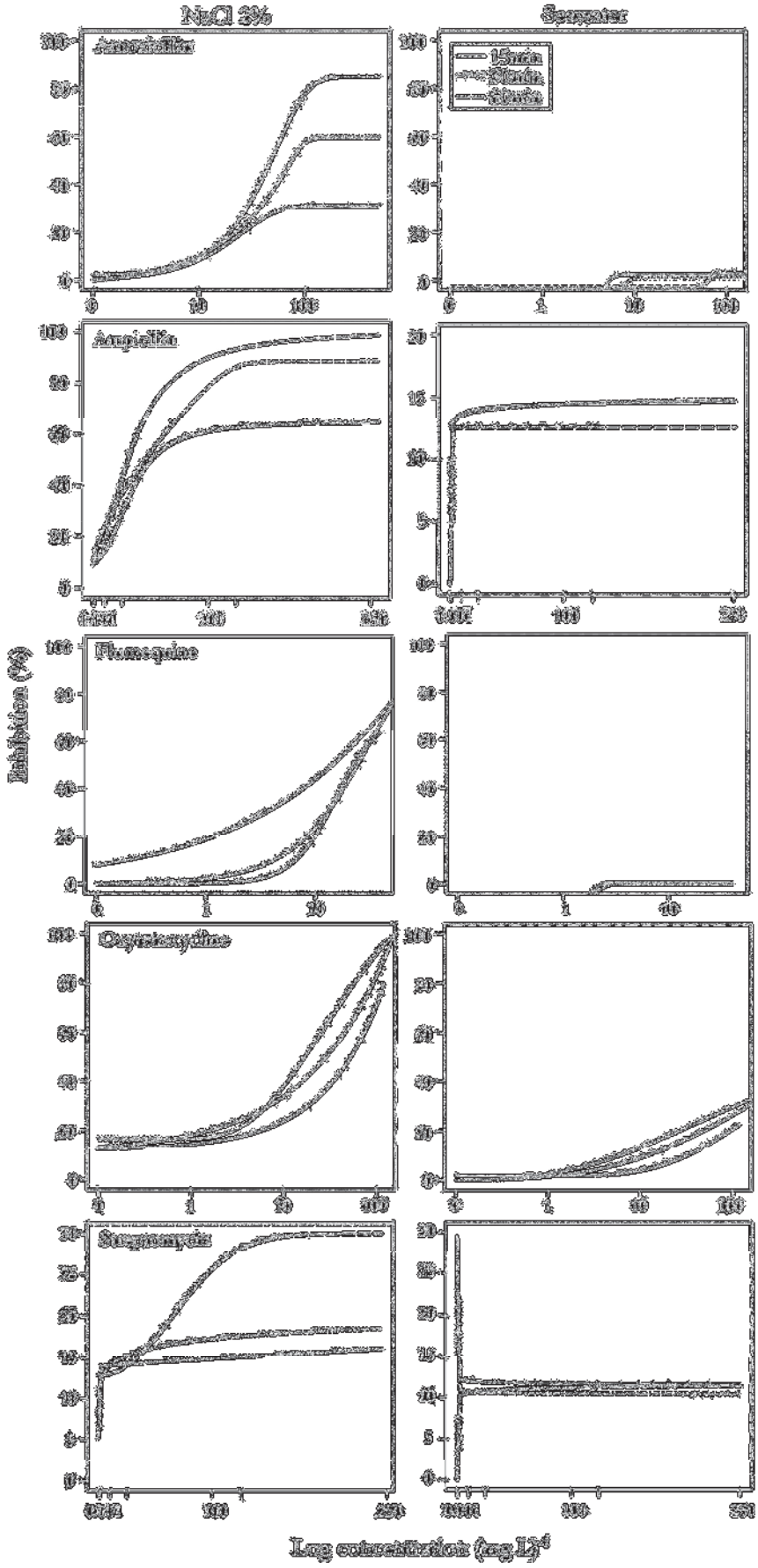


Figure 2. Concentration-response curves for the antibiotics; amoxicillin, ampicillin, flumequine, oxytetracycline and streptomycin. Different salinities and performing times are represented.

Table 1. Test endpoints at 15 minutes and at two salinities (20‰ NaCl and seawater).

20 ‰ NaCl	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	W1.4	-		-	-	-	-	-		-		50	100
Amoxicillin	W2.4	6.38	± 1.79	10.69	± 2.36	18.31	± 2.89	41.25	± 3.08	75.46	± 4.66	5	10
Ampicilin	LL.4	-		-		17.08	± 1.59	51.48	± 8.52	-		1	5
Flumequine	LL.3	2.18	± 1.26	4.19	± 3.34	-		-		-		1	2.5
Formaldehyde	LL3	0.48	± 0.06	0.87	± 0.08	1.66	± 0.12	4.95	± 0.45	14.16	± 2.19	0.5	1
Oxytetracycline	W1.4	-		-		-		15.48	± 2.09	58.92	± 41.05	5	10
Sodium hypochlorite	LL5	2.99	-	4.09	± 2.08	11.32	± 2.55	19.80	± 1.65	25.04	± 1.26	1	5
Streptomycin	W2.3	-		-		-		-		-		0.01	1
Sulphadiazine	W1.3	-		-		-		-		-		50	100

Seawater	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	LL.4	21.68	± 3.90	37.4	± 4.53	63.64	± 4.84	147.92	± 13.43	314.06	± 52.26	10	50
Amoxicillin	W2.4			-		-		-		-		-	-
Ampicilin	W2.4	-		-		-		-		-		-	-
Flumequine	W1.3			-		-		-		-		-	-
Formaldehyde	LL4	1.49		2.28		3.34		6.12		11.37		1	5
Oxytetracycline	W1.3	-		-		-		-		-		-	-
Sodium hypochlorite	W1.4	0.03	± 0.02	0.03	± 0.02	-		-		-		0.025	0.05
Streptomycin	W1.3	-		-		-		-		-		-	-
Sulphadiazine	LL.4			-		-		-		-		-	-

Table 2. Test endpoints at 30 minutes and at two salinities (20‰ NaCl and seawater).

20 ‰ NaCl	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	W1.4	-		-		-		-		-		100	-
Amoxicillin	LL5	-		-		-		35.44	± 11.80	69.52	± 7.71	-	5
Ampicillin	LL.5	-		0.21	± 0.09	6.48	± 1.16	45.41	± 3.07	-		1	5
Flumequine	LL.3	2.18	± 1.26	4.19	± 3.34	-		-		-		2.5	5
Formaldehyde	LL3	0.44	± 0.06	0.85	± 0.09	1.73	± 0.13	5.72	± 0.62	18.07	± 3.25	0.5	1
Oxytetracycline	W2.4	-		-		-		-		-		5	10
Sodium hypochlorite	LL5	-		-		-		-		25.22	± 7.62	1	5
Streptomycin	LL.4	-		-		-		-		-		1	5
Sulphadiazine	W1.3	-		-		-		-		-		50	100

Seawater	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	LL4	24.08	± 3.39	36.07	± 3.77	58.26	± 4.15	136.73	± 11.95	309.55	± 49.56	10	50
Amoxicillin	W1.3	-		-		-		-		-		2.5	5
Ampicillin	LL.3	-		51.62	± 39.05	-		-		-		-	-
Flumequine	LL4	1.14		-		-		-		-		-	-
Formaldehyde	LL4	1.06	± 0.18	1.85	± 0.22	2.95	± 0.22	6.11	± 0.28	13.01	± 1.29	0.5	1
Oxytetracycline	W1.3	-		-		50.08		-		-		-	-
Sodium hypochlorite	W1.3	0.03	± 0.01	0.03		-		-		-		0.025	0.05
Streptomycin	LL.3	-		-		-		-		-		-	-
Sulphadiazine	LL4	-		-		-		-		-		-	-

Table 3. Test endpoints at 60 minutes and at two salinities (20‰ NaCl and seawater).

20 ‰ NaCl	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	W1.4	-		45.15	± 16.31	85.36	± 30.86	196.07	± 45.5	-		100	-
Amoxicillin	W2.3	-		-		7.44	± 4.20	18.85	± 7.51	37.63	± 23.32	5	10
Ampicilin	LL.3	2.59	± 0.85	4.92	± 1.07	9.83	± 1.26	30.52	± 6.19	-		1	5
Flumequine	LL3	4.64	± 0.74	6.62	± 0.74	9.88	± 1.15	22.44	± 5.87	28.99	± 9.16	5	10
Formaldehyde	LL3	0.45	± 0.07	0.89	± 0.10	1.87	± 0.17	6.63	± 0.91	22.48	± 5.10	0.5	1
Oxytetracycline	W2.4	-		-		-		58.47		121.09		10	50
Sodium hypochlorite	W2.3	-		-		-		-		-		0.5	1
Streptomycin	W2.4	-		-		82.07	± 10.4	-		-		10	100
Sulphadiazine	W1.3	0.73		3.96		39.69		-		-		50	100

Seawater	Model	EC_5		EC_{10}		EC_{20}		EC_{50}		EC_{80}		NOEC	LOEC
Ammonium chloride	W1.3	26.48	± 1.65	36.65	± 1.81	56.25	± 3.64	140.64	± 20.76	355.81	± 86.37	10	50
Amoxicillin	W1.4	-		-		-		-		-		2.5	5
Ampicilin	LL.3	-		-		-		-		-		-	-
Flumequine	W1.4	1.41	± 8.63	-		-		-		-		1	2.5
Formaldehyde	W1.4	1.673	± 0.19	2.36	± 0.19	3.29	± 0.18	6.62	± 0.40	16.74	± 2.41	0.50	1.00
Oxytetracycline	W1.4	13.51	± 9.95	-		93.96		-		-		5	10
Sodium hypochlorite	W1.3	0.03	± 0.01	0.03	± 0.01	-		-		-		0.025	0.05
Streptomycin	LL.3	-		-		-		-		-		-	-
Sulphadiazine	LL4			-		-		-		-		-	-

On the contrary, EC_{50} from flumequine was among the highest values reported in the literature range (Isidori et al., 2005; Lalumera et al., 2004; Park and Choi, 2008). Other substances, such as amoxicillin, have shown widely different results in previous studies (Botelli et al., 2006; Park and Choi, 2008) which does not allow to draw reliable conclusions.

Ammonium chloride showed high standard deviation values due to the intrinsic unstability of this chemical (equilibrium ionized-unionized ammonia, volatilization, oxidation,…) (Källqvist and Svenson, 2003) but this substance was included because of its high presence on the surroundings of fish farms.

4.2. Effect of salinity on chemical toxicity

V. fischeri is a marine bacterium which supports a wide range of salinities. However, this test was commonly used to assess groundwater, freshwater or brackish samples by artificially adjusting the salinity to 20 ‰ NaCl. However, this standardization modifies real toxicity of samples by modifying their salinities because the salt content is directly related with toxicity (Cook et al., 2000; Girotti et al., 2002; Carballeira et al., 2011; Hernando et al., 2007). In this way, the toxicity of samples with different salinity should not be compared and can´t be extrapolated from other salinities. This study clearly proves the different toxicity of samples when changing the salinity and tries to adapt the bioluminiscent test for the assessment of marine effluents.

The protective effect that salinity has on bioassayed organisms (Cook et al., 2000) has been proved for these antibiotics and disinfectants. The higher the salinity, the lower the toxicity. On the other hand, a stimulation of bioluminescence was observed on reference samples. This stimulation might be caused by osmolarity, alkali, alkaline-earth metal ions or iron (Cook et al., 2000; Klein, 1991; Krebs, 1992), and may have masked inhibition effects. Nevertheless, seawater was artificially done so undesirable ions are not present.

On the contrary, ammonium chloride was more toxic when increasing the salinity (Table 1; Fig. 1). Although specific mechanisms of salinity interaction with non metals are largely unknown, one important factor may be the osmotic stress caused by the salinity (Cook et al., 2000) but this might have occurred in other substances. The presence of other chemicals in seawater may act as interference in the mechanisms

of action of these compounds, but this fact should not happen with artificial water (Hernando et al., 2007).

Solubility of antibiotics is generally very low, especially for flumequine and sulphadiazine which also described a higher light inhibition with increasing salinities (Fig. 1 and 2). Besides, luminescence may be stimulated by varying the salt content of samples (Cook et al., 2000, Haygood and Nealson, 1985). For all this reasons and the different bioavailability of toxicants in seawater (Peters et al., 2002) the luminiscent test protocol must be adapted for the proper assessment and comparison of marine samples.

4.3. Toxicokinetics

Toxicity measurements should be made at longer exposure times, although testing will be more onerous, because the sensitivity of test organisms to some chemicals is enhanced with increasing exposure duration (Froehner et al., 2000; Suedel et al., 1997). In addition, fish farms effluents are chronically discharged, so longer exposure times may reflect more accurately the actual situation. Thus, delayed toxicity of antibiotics may be detected (Backhaus and Grimme, 1999; Backhaus et al., 1997). This delayed toxicity has been mainly found on tested antibiotics.

5. Conclusions

The miniaturized test with *V. fischeri*, properly optimized for marine discharges, is a sensitive and cheap tool for the assessment of numerous pollutants and is also not time consuming. On the other hand, this test requires further studies on the influence of different salinities and exposure times on the luminescence of the bacteria and the toxicity of pollutants in order to be used everywhere with reliable results.

Due to the nature of fish farm discharges, chronical with low concentration of contaminants, testing is recommended with greater exposure times for the detection of delayed toxicity of some antibiotics.

In general, toxicity values were within the range of conventional tests. Nevertheless, in reference to previous studies, endpoint values obtained with the miniaturized test are usually smaller than those obtained with conventional tests.

Acknowledgements

The present study was partly financed by the Spanish Government's National Plan for Marine Culture (JACUMAR, 2008): "Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)".

References

Backhaus, T., et al , 2003. The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. Continental Shelf Research 23(17-19), 1757-1769.

Backhaus, T., Froehner, K., Altenburger, R., Grimme, L.H., 1997. Toxicity testing with *Vibrio fischeri*: a comparison between the long term (24 h) and the short term (30 min) bioassay. Chemosphere 35(12), 2925-2938.

Blaise, C., Forghani, R., Legault, R., Guzzo, J., Dubow, M.S., 1994. A bacterial toxicity assay performed with microplates, microluminometry and Microtox® reagent. BioTechniques 16(5), 932-937.

Blaise, C. 2000: Canadian application of microbiotests to asses the toxic potential of complex liquid and solid media. In: Personne, G. Jansen, CR, De Coen, W. (eds) New microbiotests for routine toxicity screening and biomonitoring. Kluwer Academic/Plenum Publishers, New York, pp 3-12.

Carballeira, C., De Orte, M. R., Viana, I. G., & Carballeira, A., 2012. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. Ecotoxicology and Environmental Safety, 78(0), 148-161.

Cook, S.V., Chu, A., Goodman, R.H., 2000. Influence of salinity on *Vibrio fishceri* and *lux*-modified *Pseudomonas fluorescens* toxicity bioassays. Environmental Toxicology and Chemistry 19(10), 2474-2477.

Cripps, S., Kumar, M., 2003. Environmental and other impacts of aquaculture, in: Lucas, J.S., Southgate, P.C. (Eds.), Aquaculture: Farming aquatic animals and plants. Blackwell Publishing, Australia.

Douet, D.G., Le Bris, H., Giraud, E., 2009. Environmental aspects of drug and chemical use in aquaculture: An overview. Options Méditerranéennes, 86, 105-12

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. JASA 50, 1096-1121.

Emmanuel, E., Keck, G., Blanchard, J.-M., Vermande, P., Perrodin, Y., 2004. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environment International, 30, 891-900.

Fiehn, O., Vigelahn, L., Kalnowski, G., Reemtsma, T., Jekel, M., 1997. Toxicity-directed fractionation of tannery wastewater using solid-phase extraction and luminescence inhibition in microtiter plates. Acta Hydrochimica et Hydrobiological, 25, 11-16.

Froehner, K., Backhaus, T., Grimme, L.H., 2000. Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. Chemosphere, 40, 821-828.

Froehner, K., Meyer, W., Grimme, L.H., 2002. Time-dependent toxicity in the long-term inhibition assay with *Vibrio fischeri*. Chemosphere, 46, 987-997.

Gellert, G., Stommel, A., 1999. Influence of microplate material on the sensitivity of growth inhibition tests with bacteria assessing toxic organic substances in water and waste water. Environmental Toxicology and Water Quality, 14, 424-428.

Girotti, S., Bolelli, L., Roda, A., Gentilomi, G., Musiani, M., 2002. Improved detection of toxic chemicals using bioluminescent bacteria. Analytica Chimica, 471, 113-120.

Huntington, T.C., 2006. Some Aspects of the environmental impact of aquaculture in sensitive areas. Report to the DG Fish and Maritime Affairs of the European Commission.

Hernando, M.D., De Vettori, S., Martínez-Bueno, M.J., Fernández-Alba, A.R., 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere, 68, 724-730.

Hirmann, D., Loibner, A.P., Braun, R., Szolar, O.H.J., 2007. Applicability of the bioluminescence inhibition test in the 96-well microplate format for PAH-solutions and elutriates of PAH-contaminated soils. Chemosphere, 67, 1236-1242.

Holten Lützhøft, H.-C., Halling-Sørensen, B., Jørgensen, S.E., 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Archives of Environmental Contamination And Toxicology, 36, 1-6.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of the Total Environment 346, 87-98.

ISO 11348-3, 2007. Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) - Part 3: Method using freeze-dried bacteria.

Jennings, V.L.K., Rayner-Brandes, M.H., Bird, D.J., 2001. Assessing chemical toxicity with the bioluminescent Photobacterium (*Vibrio fischeri*): a comparison of three commercial systems. Water Resources, 35(14), 3448-3456.

Källqvist, T., Svenson, A., 2003. Assessment of ammonia toxicity in tests with the microalga, *Nephroselmis pyriformis*, Chlorophyta. Water Research, 37, 477-484.

Koutny, M., Zaoralkova, L., 2005. Miniaturized kinetic growth inhibition assay with denitrifying bacteria *Paracoccus denitrificans*. Chemosphere, 60, 49-54.

Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., Fanelli, R., 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere, 54, 661-668.

Lorenzo, J.I, Nieto, O., Beiras, R. (2002). Effect of humic acids on speciation and toxicity of copper
to *Paracentrotus lividus* larvae in seawater. Aquatic Toxicology, 58, 27-41.

Maxam, G., Hahn, S., Dott, W., Eisentraeger, A., 2002. Assessment of the influence of use on ecotoxicological characteristics of synthetic ester lubricants. Ecotoxicology, 11, 349-355.

Muñoz, I., Martínez-Bueno, M.J., Agüera, A., Fernández-Alba, A.R., 2010. Environmental and human health risk assessment of organic micro-pollutants occurring in a Spanish marine fish farm. Environmental Pollution, 158,1809-1816.

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology, 17, 526-538.

Paixao, S. M., Silva, L., Fernández, A., O'Rouke, K., Mendonça, E., Picado, A., 2008. Performance of a miniaturized algal bioassay in phytotoxicity screening. Ecotoxicology, 17,165-171.

Parvez, S., Venkataraman, C., Mukherji, S., 2006. A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environment International, 32, 265-268.

Pavlić, Ž., Stjepanović, B., Horvatić, J., Peršić, V., Puntarić, D., Čulig, J., 2006. Comparative sensitivity of green algae to herbicide using erlenmeyer flask and microplate growth inhibition assays. Bulletin of Environmental Contamination and Toxicology, 76, 883-890.

Peters, C. Becker, S. Noack, U. Pfitner,S. Bülow, W. Barz, K. Ahlf, W. and Berghahn, R. (2002): A marine bioassay test set to asses marine water and sediment quality- its need, the approach and first results. Ecotoxicology, 11(5), 379-384.

Pouliquen, H., Delépée, R., Larrhantec-Verdier, M., Morvan, M., Le Bris, H., 2007. Comparative hydrolisis and photolysis of four antibacterial agents (oxytetracycline, oxolinic acid, flumequine and florfenicol) in deionised water, freshwater and seawater under abiotic conditions. Aquaculture, 262, 23-28.

R Development Core Team, 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org.

Reemtsma, T., Putschew, A., Jekel, M., 1999. Industrial wastewater analysis: a toxicity-directed approach. Waste Management, 19, 181-188.

Rey-Asensio, A., Carballeira, C., Viana I.G., Carballeira, A. (2011). Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioacumulación de microcontaminantes. Actas Foro dos Recursos mariños e da Acuicultura das Rías galegas, 13: 211-228.

Ricco, G., Tomei, M.C., Ramadori, R., Laera, G., 2004. Toxicity assessment of common xenobiotic compounds on municipal activated sludge: comparison between respirometry and Microtox®. Water Research, 38(8), 2103-2110.

Ritz, C., Streibig, J. C., 2005. Bioassay Analysis using R. J. Statist. Software, Vol 12, Issue 5.

Schmitz, R.P.H., Eisenträger, A., Dott, W., 1998. Miniaturized kinetic growth inhibition assays with *Vibrio fischeri* and *Pseudomonas putida* (application, validation and comparison). Journal of Microbiological Methods, 31, 159-166.

Schmitz, R.P.H., Eisenträger, A., Dott, W., 1999. Agonistic and antagonistic toxic effects observed with miniaturized growth and luminescence inhibition assays. Chemosphere, 38(1), 79-95.

Shahidul, I., Tanaka, M., 2004. Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Marine Pollution Bulletin, 48, 624-649.

Suedel, B.C., Rodgers, J.H., Deaver Jr., E., 1997. Experimental factors that may affect toxicity of cadmium to freshwater organisms. Archives of Environmental Contamination and Toxicology, 33, 188-193.

Tacon, A.G.J., Phillips, M.J., Barg, U.C., 1995. Aquaculture feeds and the environment: the Asian experience. Water Science and Technology, 31(10), 41-59.

Tamminen, M.V., Virta, M.P.J., 2007. Quantification of ecotoxicological tests based on bioluminescence using Polaroid film. Chemosphere, 66, 1329-1335.

Vighi, M., Migliorati, S., Monti, G.S., 2009. Toxicity on the luminescent bacterium *Vibrio fischeri* (Beijerinck). I: QSAR equation for narcotics and polar narcotics. Ecotoxicology and Environmental Safety, 72, 154-161.

Wolska, L., Sagajdakow, A., Kuczyńska, A., Namieśnik, J., 2007. Application of ecotoxicological studies in integrated environmental monitoring: Possibilities and problems. Trends in Analytical Chemistry, 26(4), 332-344.

Zaroogian, G.E., Pesch, G., Morrison, G., 1969. Formulation of an artificial sea water media suitable for oyster larvae devlopment. American Zoologist, 9. Abstact No 549.

Ecotoxicology and Environmental Safety 78 (2012) 148–161

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms

C. Carballeira [a,*], M.R. De Orte [a], I.G. Viana [b], A. Carballeira [c]

[a] Departamento de Química Física, Cátedra UNESCO/UNITWIN/WICOP, Facultad de Ciencias del Mar y Ambientales, Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain
[b] Instituto Español de Oceanografía, Centro Costero de A Coruña, 130, 15080, A Coruña, Spain
[c] Departamento de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, A Coruña, Spain

ARTICLE INFO

Article history:
Received 2 September 2011
Received in revised form
3 November 2011
Accepted 16 November 2011
Available online 2 December 2011

Keywords:
Pisciculture discharges
Pond
Biomonitoring
Toxicity test
Microtox®
Microalgal growth
Sea urchin larval development

ABSTRACT

Environmental monitoring plans (EMP) that include chemical analysis of water, a battery of bioassays and the study of local hydrodynamic conditions are required for land-based marine aquaculture. In this study, the following standardized toxicity tests were performed to assess the toxicity of effluents from eight land-base marine fish farms (LBMFFs) located on the northwest coast of Spain: bacterial bioluminescence (with *Vibrio fischeri* at 15 and 30 min), microalgal growth (with *Phaeodactyllum tricornutum* and *Isochrysis galbana*) and sea urchin larval development (with *Paracentrotus lividus* and *Arbacia lixula*). These bioassays were evaluated for inclusion in routine fish farm monitoring. Effective concentrations (EC_5, EC_{10}, EC_{20}, EC_{50}) for each bioassay were calculated from dose–response curves, obtained by fitting the bioassay results to the best parametric model. Moreover, a graphical method of integrating the results from the battery of bioassays and classifying the toxicity was proposed, and the potential ecotoxic effects probe (PEEP) index was calculated. The bacterial bioluminiscence test at 30 min, growth of *I. galbana* and larval development of *A. lixula* were found to be the most sensitive and useful tests. Graphical integration of these test results enabled definition of the ecotoxicological profiles of the different farms. The PEEP index, considering EC_{20}, efficiently reflected the toxic loading potential of LBMFF effluents. In conclusion, a battery of bioassays with species from different low trophic levels is recommended as a rapid and cost-effective methodology for assessing LBMFF discharges. The graphical integration method and the PEEP index are proposed for consideration in EMPs for such farms.

1. Introduction

Marine land-based aquaculture has become more intensive over the last 15 years, mainly as a result of the introduction of new technologies, expansion of suitable sites, improvements in feed technology, improved understanding of the biology of the species farmed, increased water quality within farming systems and the increased demand for fish products (Read and Fernandes, 2003). Although the output from fisheries remained constant, flatfish aquaculture increased significantly from 26,300 t in 2000 to 148,800 t in 2008, with China and Spain being the leading producers (FAO, 2010). In Spain, the main species involved is the turbot (*Psetta maxima*), which is produced in land-based facilities.

Fish production can generate considerable amounts of effluents, such as waste food, feces, medications and pesticides, which may have (eco)toxic and trophic effects (eutrophization), and consequently may alter the structure and development of the receiving ecosystems. Discharges from aquaculture to the aquatic environment may be categorized as continuous discharge, periodic discharge and pulses of veterinary medicines and/or their mixtures (Tello et al., 2010). Discharges include a wide range of chemicals involved in feed composition and metabolization, liming materials, algicides, disinfectants, antibiotics, hormones, osmoregulators and probiotics. Most studies of effluents from land-based fish farms have focused on output nutrients, biochemical oxygen demand and suspended solids, but few studies have considered chemicals and pathogens (Tello et al., 2010).

If the understanding of the ecotoxicological effects does not keep pace with the emergence of new aquaculture products, waste water discharges from fish farms may become a problem. A serious environmental impact may occur when untreated effluents are released into the sea. Regulations, methodological guidelines and protocols, and monitoring plans have been developed for marine fish farms (Fernandes et al., 2000; GESAMP, 1996; Hansen et al., 2001; Roque D'Orbcastel et al., 2004; SEPA, 1999; Stigebrandt et al., 2004), although these all refer to the environmental impact caused by marine fish farms installed in

* Corresponding author. Fax: +34 956016040.
E-mail addresses: carlos.carballeira@uca.es (C. Carballeira), manoela.romano@uca.es (M.R. De Orte), inesgviana@gmail.com (I.G. Viana), alejo.carballeira@usc.es (A. Carballeira).

0147-6513/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2011.11.022

cages. Protocols or environmental monitoring plans (EMPs) for land-based marine fish farms (LBMFFs) are practically non-existent, and there is a critical need to improve the information on inland fishery resources and on the people that use and depend on them (FAO, 2010). Since monitoring of the impacts of cage fish culture mainly focuses on benthic communities, which are largely affected by the type of site where the cages are located and the type of management carried out, other options for the design of EMPs for LBMFFs must be considered.

To date, the organization responsible for the environmental surveillance of LBMFFs in Galicia (northwest Spain) has only considered conventional physicochemical parameters in monitoring the effluents. The information obtained from such analyses is insufficient, because emerging pollutants are not taken into account, and the data do not indicate the potential effects on ecological processes in the water column (Sarà, 2007) because they do not reflect the bioavailability of contaminants (Hernando et al., 2007) and pollutant interactions (e.g. synergistic effects) (Richardson et al., 2007). Furthermore, chemical analysis of trace elements is complex and entails high costs. Evaluation of the relationship between the information obtained by monitoring and the cost of obtaining such information (Borja, 2002) within the *experimental control* versus *environmental realism* concept are necessary when designing a practical EMP. The information compiled should describe the ecological processes and not merely describe local situations (Underwood, 1997).

In this context, the use of laboratory bioassays to evaluate the potential toxicity of fish farm effluents, in combination with study of the hydrodynamic conditions in the area, may provide useful ecotoxicological data, and may also enable estimation of the capacity of the environment to assimilate the impact. Field testing and field monitoring provide more realistic ecological evidence, but cannot always be applied, because of their complexity and high cost (DelValls, 2007). Laboratory bioassays are monitoring tools that try to estimate the likelihood that contaminants within an environmental matrix will cause effects at different levels of organization and, ultimately, harm the surrounding ecosystem. Laboratory bioassays are relatively simple to carry out and therefore should be considered in EMPs with the same frequency as conventional chemical analyses.

Many regulatory agencies assess the environmental toxicity of discharges by use of a battery of test organisms that may include aquatic invertebrates, fish, bacteria, microalgae and higher plants (OECD, 1998; USEPA, 2000). A meaningful battery of bioassays with high sensitivity to pollutants for the evaluation of effluents and superficial waters should ideally include test species belonging to different trophic levels: producers (algae and higher plants), consumers (crustaceans and rotifers) and decomposers (Mankiewicz-Boczek et al., 2008) so that the toxicological profile will be better understood. The selection of test species is determined by their relevance, prevalence, accessibility, ease of maintenance and culture, cost-effectiveness and by how easy they are to observe and quantify (Jiangning et al., 2004).

The bioluminescence inhibition test that uses the bacteria *Vibrio fischeri* has been widely applied in water analysis and to evaluate the toxicity of biocides, which are the same or similar to those used in fish farming (Backhaus and Grimme, 1999; Coelho et al., 2011; Hernando et al., 2007; Isidori et al., 2005; Lalumera et al., 2004; Muñoz et al., 2010; Park and Choi, 2008). Microalgae have been recommended as test organisms because of their ecological relevance and sensitivity (Pavlic et al., 2006; Satoh et al., 2005). Toxicity tests conducted with embryos of invertebrates are widely used because of their sensitivity to chemicals of different nature, ease of handling, low cost and applicability in both laboratory and field conditions (Beiras et al., 2001).

According to this, the first aim of this study was to apply a set of marine bioassays to evaluate the impact of LBMFFs on marine life. This set of tests comprised the bioluminescence inhibition test with the bacterium *V. fischeri*, the embryo development test with the sea urchin species *Paracentrotus lividus* and *Albacia lixula*, and the microalga growth test with the species *Phaeodactyllum tricornutum* and *Isochrysis galbana*. A minimal battery of bioassay was thus proposed for assessment of the effects of discharges from LBMFFs on decomposers (bacteria), primary producers (microalgae) and primary consumers (sea urchins). Because of the lack of knowledge of the impact generated by this type of aquaculture, the use of two different species of microalgae and sea urchins and different exposure times with *V. fischeri* were tested to determine the most suitable assay in accordance with the physicochemical conditions of each farm.

The second objective was to propose, through prior evaluation, a toxicity classification system based on the aforementioned battery of bioassays for routine integral biological monitoring of LBMFFs.

2. Material and methods

2.1. Effluent characteristics

This study focused on the effluents generated by eight LBMFFs (I, II, III, IV, V, VI, VII and VIII) located in Galicia (northwest Spain; Fig. 1). These farms grow *Psetta maxima* and *Solea solea* and they account for approximately the 50% of the production of these species in Spain (APROMAR, 2011). Significant differences in the level of production and the volume of residual water generated can be found amongst each other (Table 1).

The Galician agency responsible for the environmental monitoring of the LBMFFs provided the physicochemical characterization of the effluents from the fish farms (Table 1). The same characteristics were measured in the input (I) and in the output (O) water from 18 LBMFFs situated in the same region during the period 2002–2008, and the average values obtained for the samples were similar to those obtained in the effluents tested (Table 2).

The presence of specific contaminants was evaluated by means of bioaccumulation measurements in several native macroalgae (*Fucus* sp. and *Codium tomentosum*) and in transplanted specimens of the macroalga *Saccharina saccharina* (Rey-Asensio et al., 2010). Metals are major constituents of disinfectants and anti-fouling products and are present in the diet of cultivated fish (Dean et al., 2007). There were no significant differences ($p < 0.05$) in the bioaccumulation of the most important metals (Cd, Cr, Cu, Pb and Zn) in either of the species within a gradient of sampling sites affected by LBMFF discharges (Rey-Asensio et al., 2010). All metal concentrations were below the mean background concentration determined for meso-polyhaline waters (Tueros et al., 2008). The concentrations of the antibiotics sulfadiazine, flumequine, oxolinic acid, oxytetracycline and amoxicillin were found to be below the detection limit of chemical analysis in all species, but significant differences ($p < 0.05$) in their bioconcentration were observed in *S. saccharina* collected in the surroundings of the LBMFFs. Some pesticides such as prometryn, prometon and chlorothalonil were also detected in the transplanted algal specimens (Rey-Asensio et al., 2010).

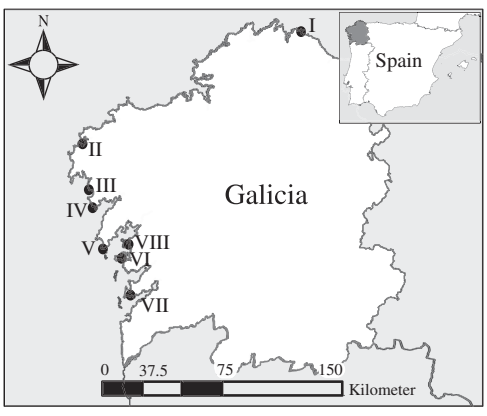


Fig. 1. Location of the eight land-based marine fish farms (designated from I to VIII) on a map of Galicia (northwest Spain).

Table 1
Physicochemical characteristics of effluents sampled at the land-based marine fish farms (LBMFFs) under study.

LBMFF	Production (t year^{-1})	Salinity (gL^{-1})	pH	O_2 (mgL^{-1})	O_2 (%)	SS (mgL^{-1})	TOC (mgL^{-1})	PO_4 (mgL^{-1})	NO_2 (mgL^{-1})	NO_3 (mgL^{-1})	NH_3 (mgL^{-1})
I	2250	32.4	7.48	8.3	79.6	23	5.64	0.22	0.086	0.190	0.85
II	292	34.7	7.92	8.4	80.1	17	5.35	0.42	0.072	0.200	0.45
III	308	34.8	7.94	8.5	82.2	18	7.05	0.25	0.069	0.192	0.63
IV	1194	34.7	7.63	7.8	79.1	21	8.04	0.29	0.080	0.205	0.78
V	348	34.2	7.80	8.9	87.3	16	6.09	0.24	0.078	0.141	0.56
VI	44	34.5	7.64	8.0	79.5	18	2.36	0.21	0.075	0.536	0.57
VII	285	34.3	7.59	8.2	80.3	14	4.70	0.23	0.066	0.206	1.04
VIII	189	30.3	7.71	7.9	78.4	39	7.56	0.47	0.044	0.287	0.36

Physicochemical characterization included yearly production, effluent salinity, pH, dissolved oxygen (O_2), suspended solids (SS), total organic carbon (TOC), phosphates (PO_4), nitrites (NO_2), nitrates (NO_3) and ammonia (NH_3).

2.2. Effluent sampling

Water samples were collected in October 2008 at the output of eight LBMFFs located in NW Spain, dedicated to turbot culture (*Psetta maxima* L.) and fattening of sole (*Solea solea* L.), and in which production levels are different (Table 1). All the farms under study have open water circulatory systems, except fish farm VIII, which has a water recycling system.

Sampling was performed during the period of maximum productivity (September–October 2008) in order to reflect the highest potential impact of the farms. Representative samples of effluent were obtained by programming a peristaltic pump (Gilson M312), placed at the output channel of each farm, to obtain a sample of about 6 L of effluent (before being diluted with receiving seawater) during the hours when fish are metabolically most active (8 a.m. to 8 p.m.). Effluent samples were obtained 48 h before the beginning of the experiment.

2.3. Toxicity tests

2.3.1. Dilution preparation

Effluent dilutions were prepared in artificial sea water (Lorenzo et al., 2002), except for those in the bioluminescence inhibition test, for which a 2% NaCl solution was used (Microtox® commercial test kit, Azur Environmental). Dilutions were expressed as the percentage of effluent, as follows: 0% (control; artificial seawater only), 5%, 25%, 50%, 75% and 100% (undiluted effluent).

2.3.2. Bioluminescence test with bacteria

The bioluminescence test with the bacterium *V. fischeri* measures the reduction in luminescence emitted by the bacteria when exposed to a contaminated matrix (strain NRRL-B-11177). The Microtox® commercial test kit (Azur Environmental, 1998) was used and the protocol was adapted as described by Hirmann et al. (2007). Three replicates were prepared for each sample, and eight for the control with the reference solution (2% NaCl). Phenol was used as the standard to guarantee the validity of each well and the test. Replicates were considered valid only when coefficients of variation were lower than 15%. Bioluminescence was measured with a DTX 880 Multidetector after incubation times of 15 and 30 min. The percentage inhibition of luminescence was defined for each effluent dilution, as follows: $INH\ (\%)=[(I_c-I_t)/I_c]100$, where I_c and I_t are the luminescence of the control and the test sample, respectively.

2.3.3. Microalgal growth test

Phaeodactylum tricornutum (AROSA) and *Isochrysis aff. galbana* (Clon T-ISO) were obtained from the algae collection belonging to the Department of Microbiology and Parasitology (University of Santiago de Compostela). Microalgae were pre-cultured in sterilized natural seawater enriched with ALGAL medium (Fabregas et al., 1984) in Erlenmeyer flasks. The flasks were fitted with transpirable tops, shaken once a day (to ensure adequate aeration) and maintained at 22 °C under a photoperiod of 16 h of light and 8 h of darkness and irradiated at 160 μEm^{-2} s^{-1}. The environmental conditions were in accordance with international standards (ISO 10253, 2006) for such tests.

The *ALGAL* medium was used in the toxicity tests. It was diluted six times in order to allow a population growth of at least 0.04 h^{-1}, according to the ISO protocol. This would minimize any interference of the medium in the toxicity values (De Orte et al., 2009). Microalgae were inoculated in diluted media during exponential growth. All tests were performed in 96 well flat bottom microplates (Sero-Wel Bibby Sterelin Ltd, Stone, Staffs, UK). The initial cell density in the microplates was 10^5 cell mL^{-1} and the plates were maintained under the same conditions as the pre-cultures. The volume of the test solution was 300 μL. Reference density measurements were made in a Neubauer chamber in an optical microscope (OLYMPUS CKX41).

Algae growth was monitored every 24 h for 4 days (96 h) by measuring the absorbance of the growth medium (450 nm) in a microplate reader (BECKMAN COULTER DTX 880 multimode detector). Growth inhibition was determined in each sample by comparing, every 24 h, the cell density with that of the control samples (three replicates with sterilized natural seawater).

2.3.4. Sea urchin larval development test

Adult sea urchins of both species were collected by scuba diving, in an intertidal zone at a clean site on the Galician coast. In the laboratory, the specimens were maintained in aerated pools at 15 °C for acclimation during 10 days. Gametes were obtained by injecting 1 mL of KCl (0.5 M) through the perioral membrane of a pair of adults. *In vitro* fertilization was conducted following the methodology described by Fernández and Beiras (2001). Incubation vials of 20 mL volume were filled with the effluent dilution series (Section 2.3.1), in quadruplicate. Solutions of zinc sulfate and ammonium chloride were used as standards to verify the validity of the test. Approximately 400 fertilized eggs were placed in each vial. The vials were incubated for 48 and 72 h at 20 °C for *P. lividus* and *A. lixula*, respectively. After the incubation period, larval development was stopped and the larvae were fixed by adding a drop of 40% formalin. The salinity and pH of all samples were adjusted within the optimal range for each species (Aguirre-Martínez et al., 2011). The endpoint measured in each vial was expressed as the percentage of abnormal larvae. One hundred larvae were counted in an optical microscope (OLYMPUS CKX41). Abnormal larvae included all pre-larval stages and larvae that displayed anomalies in shape and skeleton, according to the skeletal abnormality criteria reported by Carballeira et al. (2010).

2.4. Statistical analysis

2.4.1. Dose–response curves

The Drc Package (Ritz and Streibig, 2005) under R (R Development Core Team, 2008) was used to calculate the dose–response relationships. For each effluent, the most suitable model was selected from a pool of different parametric models. The toxic effects were calculated from the model equation and, where possible, expressed as effective concentrations (EC_5, EC_{10}, EC_{20} and EC_{50}). Confidence intervals were calculated from the *t*-distribution and the delta method (asymptotic-based estimations).

Differences between dilutions and reference samples were determined for each effluent by application of a post hoc Dunnett test (ANOVA) under SPSS software (version 17.0). The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined from the results of these tests. Dilutions were categorized according to their statistical significance as a ($p<0.001$), b ($p<0.01$) or c ($p<0.05$).

2.4.2. Minimal biological test battery

The aim of this study was to establish a minimal and relevant biological test set, and therefore the most sensitive species were chosen. The dilution that best explained the variability between farms (i.e. the "preferred dilution") was selected, by ANOVA of the whole data and comparison of pairs of bioassays. The selection of only one dilution may reduce the cost and time required for environmental assessment of discharges from the LBMFFs.

The Mann–Whitney *U*-test and a Student's *t*-test were used to compare the homogeneity of variance in the data for times of exposure (bacteria) and pair of species (microalgae and sea urchins) in order to test for differences in sensitivity between species or times. The endpoints at the "preferred dilution" for each pair of species were determined by nonparametric Kernel density estimation with the Gaussian function. The most sensitive species from each trophic level were selected by determining the effective concentrations (from dose–response curves) and Kernel density functions. For these analyses, the add on MASS package under Kernel Density Estimation Software (v1.1.23-r6) (Wessa, 2008) and SPSS 17.0 were used. Differences were considered significant at $p<0.05$.

Once the preferred dilution and most sensitive species were identified, the endpoints were integrated and represented in a triaxial diagram.

Table 2
Average physicochemical characteristics (period 2002–2008) of the input (I) and output (O) water from eighteen land-based marine fish farms (LBMFFs) on the Galician coast (NW Spain). Physicochemical characterization included effluent salinity, pH, dissolved oxygen (O_2), suspended solids (SS), total organic carbon (TOC), phosphates (PO_4), nitrites (NO_2), nitrates (NO_3) and ammonia (NH_3).

	Salinity (gL^{-1})		pH		O_2 (mgL^{-1})		O_2 (%)		SS (mgL^{-1})		TOC (mgL^{-1})		PO_4 (mgL^{-1})		NO_2 (mgL^{-1})		NO_3 (mgL^{-1})		NH_3 (mgL^{-1})	
	I	O	I	O	I	O	I	O	I	O	I	O	I	O	I	O	I	O	I	O
Average	34.3	34.5	7.98	7.7	8.5	8.3	85.9	84.2	13	18	6.06	7.63	0.18	0.24	0.041	0.075	0.170	0.198	0.31	0.56
± SD	0.4	0.4	0.48	0.61	0.4	0.2	7.1	4.6	16	12	3.02	1.94	0.05	0.04	0.011	0.016	0.041	0.022	0.12	0.42
n	179	179	179	179	162	162	162	162	179	179	103	103	143	143	179	179	24	24	19	19

Physicochemical characterization included effluent salinity, pH, dissolved oxygen (O_2), suspended solids (SS), total organic carbon (TOC), phosphates (PO_4), nitrites (NO_2), nitrates (NO_3) and ammonia (NH_3).

2.5. Potential ecotoxic effects of effluents from LBMFF

The PEEP index developed by Blaise and Férard (2005) provides comprehensive data from biological testing, as such data are normally scattered and difficult to find. The index enables assessment and comparison of the toxic potential of wastewater effluents as a single numerical value that integrates both its toxic potential (determined by a battery of small-scale bioassays representing different biological levels and types of toxic effects) and its flow. This index is appropriate for the assessment of effluents from LBMFFs, which have relatively low toxicity. The PEEP is usually calculated from NOEC and LOEC values, which cannot always be obtained (Isnard et al., 2001), so that the index was calculated with EC_{10} and EC_{20} values. Toxicological parameters were first transformed into toxic units (TU), by use of the formula $TU = C/EC_x$, where C is the maximum effluent concentration used (in this case 100%). The PEEP index was then calculated as follows:

$$\mathrm{PEEP} = \log_{10}\left[1 + n\left(\sum \frac{T_i}{N}\right)Q\right]$$

where $[n(\sum T_i/N)]$ is the toxic print, $[n(\sum T_i/N)Q]$ is the toxic loading, n is the number of biotests exhibiting a toxic response, T_i is the number of TU generated by each biotest (i) at the effluent samples, N is the maximum number of measurable responses and Q is the effluent flow (m^3h^{-1}).

3. Results

Performance of the different bioassays was validated according to standard requirements. The internal quality control data for phenol are consistent with the recommended EC_{50} value range of 13.00–26.00 mg L^{-1} (Azur Environmental, 1998), indicating an adequate physiological state of the tested microorganisms, according to the *V. fischeri* protocol. The growth rate of control specimens of both species of microalgae tested was within the range required in the ISO protocol. Both sea urchin species showed less than 10% of abnormal larvae after exposure to the reference samples and EC_{50} values corresponding to the standard solutions were consistent with those reported by Cesar et al. (2004).

Dose–response curves (Figs. 2–4) obtained by parametric modeling enabled calculation of more than half of the total EC values (Table 3). The small standard errors associated with the mean EC values for *V. fischeri* and sea urchin bioassays confirmed the low variation in these bioassays and indicated good precision and consistency. On the contrary, wide confidence intervals were observed for the microalgal bioassay, especially when *P. tricornutum* was used and/or when percentages of growth inhibition were not affected by increasing dilution (e.g. fish farms I, II, IV and VII).

3.1. Bioluminescence inhibition test

The percentage reduction in bioluminescence, measured after 15 and 30 min of exposure, was weakly related to the effluent dilutions (Fig. 2). Although significant differences ($p < 0.05$) from the control were found at the highest dilutions (5% effluent), the reduction in bioluminescence remained below 20%, even with 100% of effluent from farms I, VI and VII, and barely surpassed 50% in any case, except for bacteria exposed to effluent V. It was not possible to calculate the EC_5 and the EC_{50} in many cases, and the EC_{50} was often estimated from the model fit (Table 3). The comparison between EC values measured at 15 and 30 min showed that the sensitivity of *V. fischeri* increased with increasing exposure time, in at least 83% of cases. Nevertheless, these differences were not significant ($p > 0.05$) (Table 4). The highest toxicity, expressed as the lowest EC_{10} and EC_{20} values at 30 min, was observed with effluents VIII and V, while the lowest toxicity, expressed as the highest values, was observed with effluents I, IV and VII.

3.2. Microalgal growth test

Microalgal growth tests indicate two types of effects, toxic and trophic, depending on whether growth is inhibited (toxic effect)

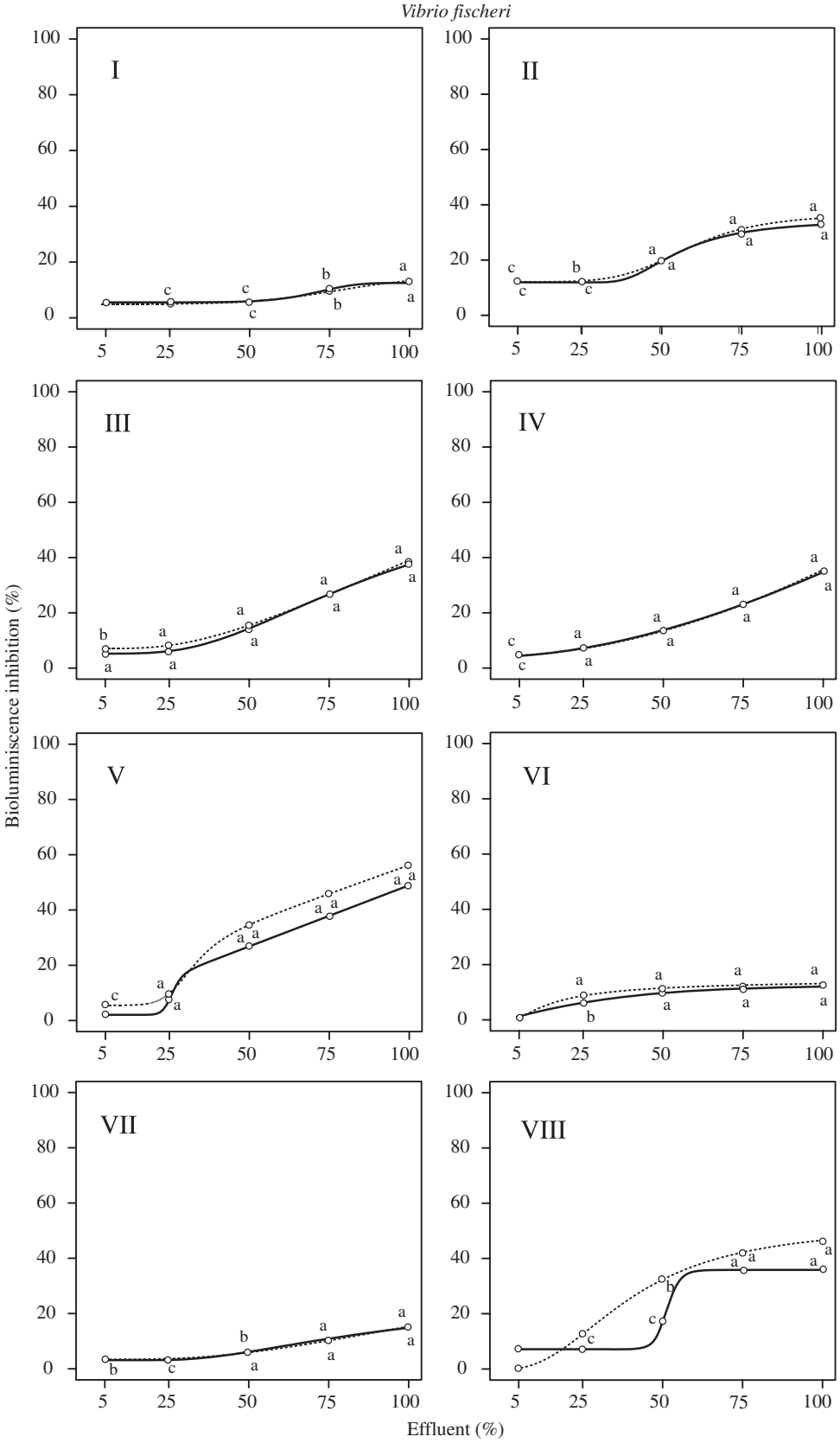


Fig. 2. Dose–response curves obtained with the *Vibrio fischeri* bioluminescence bioassay for each effluent after incubation times of 15 min (solid line) and 30 min (dashed line). Significant differences between dilutions and control are indicated as follows: a ($p < 0.001$), b ($p < 0.01$) and c ($p < 0.05$).

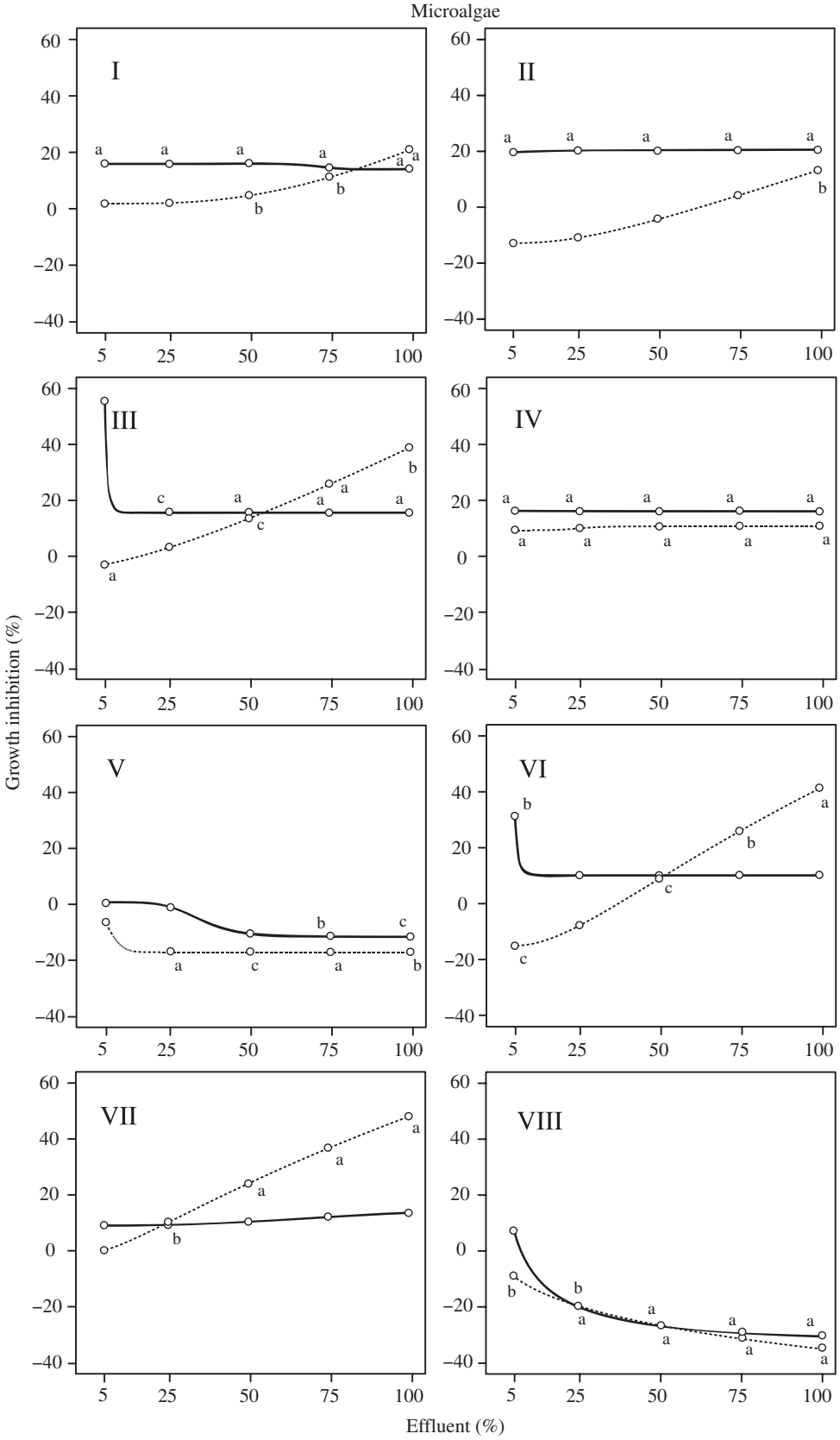


Fig. 3. Dose–response curves obtained for each effluent with the microalga growth test with the species *Phaeodactylum tricornutum* (solid line) and *Isochrysis aff. galbana* (dashed line). Significant differences between dilutions and control are indicated as follows: a ($p < 0.001$), b ($p < 0.01$) and c ($p < 0.05$).

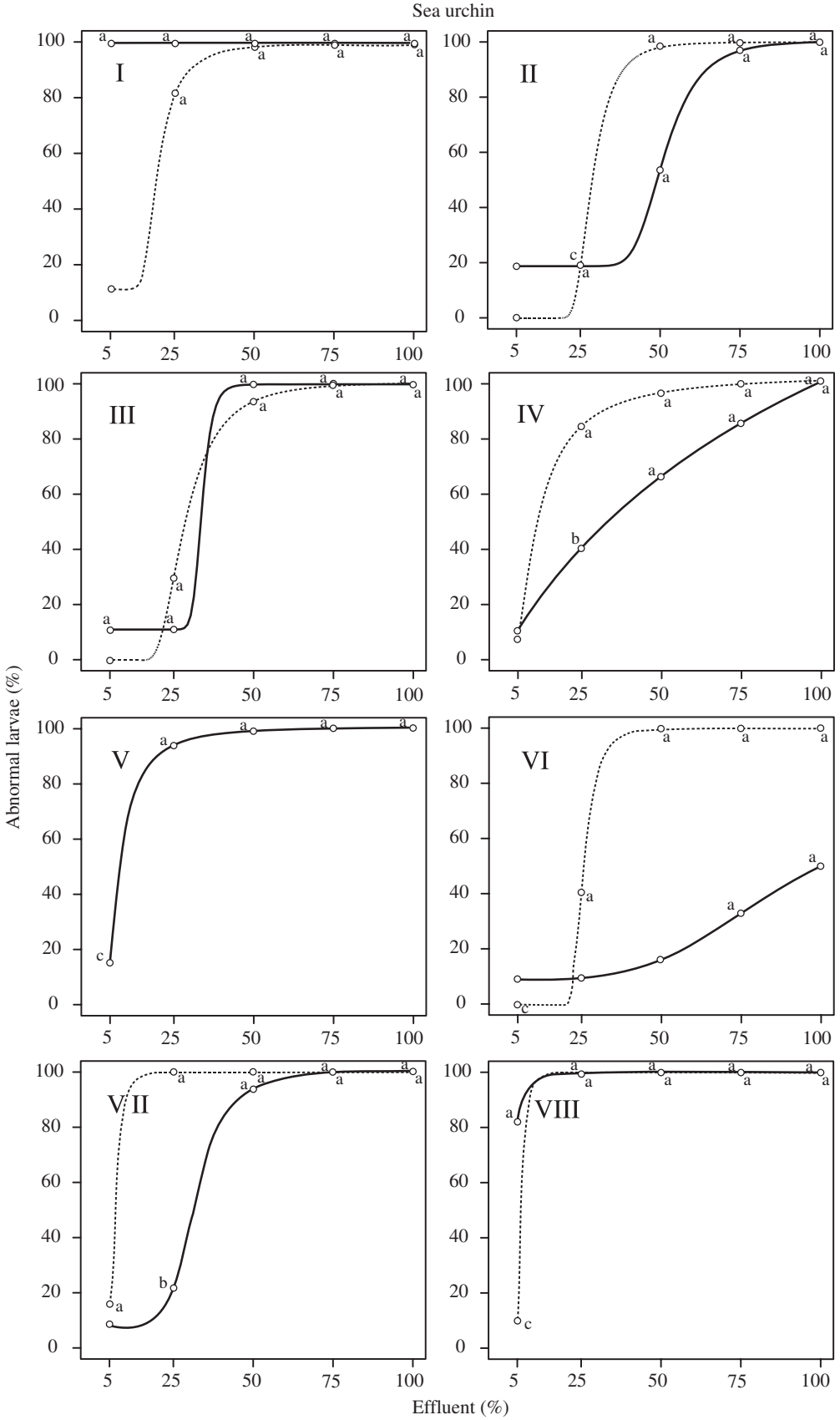


Fig. 4. Dose–response curves obtained for each effluent with the sea urchin embryo development test with the species *Paracentrotus lividus* (solid line) and *Arbacia lixula* (dashed line). Significant differences between dilutions and the control are indicated as follows: a ($p < 0.001$), b ($p < 0.01$) and c ($p < 0.05$).

Table 3
Serial effective concentrations (EC_x) of LBMFF effluents obtained with the bacterial bioluminescence test, the microalgal growth test and the sea urchin embryo development test.

LBMFF	Test species	EC_5	EC_{10}	EC_{20}	EC_{50}	NOEC	LOEC	EC_{10}/NOEC
I	*V. fischeri* (15)	n.d.	72.65 (±9.96)	n.d.	n.d.	5	25	14.6
	V. fischeri (30)	n.d.	76.73 (±108.23)	158.82 (±478.85)	n.d.	25	50	3.1
	P. tricornutum	n.d.	n.d.	n.d.	n.d.	n.d.	5	n.d.
	I. galbana	48 (±69.84)	69.18 (±99.51)	96.34 (±139.38)	153.94 (±229.85)	25	50	2.8
	P. lividus	<5	<5	<5	<5	n.d.	5	n.d.
	A. lixula	3.98 (±0.56)	4.83 (±0.6)	6.25 (±0.69)	11.32 (±1.49)	5	25	1.0
II	*V. fischeri* (15)	n.d.	n.d.	51.08 (±4.23)	n.d.	n.d.	5	n.d.
	V. fischeri (30)	n.d.	n.d.	50.59 (±5.84)	n.d.	n.d.	5	n.d.
	P. tricornutum	1.34E−26 (±6.28E−25)	5.59E−15 (±15E−13)	10.06 (±153.19)	n.d.	n.d.	5	n.d.
	I. galbana	80.97 (±107.79)	91.24 (±133.57)	109.59 (±183.06)	159.61 (±335.6)	75	100	1.2
	P. lividus	n.d.	n.d.	37.59 (n.d.)	49.30 (±0.53)	5	25	n.d.
	A. lixula	22.79 (±0.46)	23.76 (±0.29)	25.13 (±0.18)	28.69 (±0.85)	5	25	4.8
III	*V. fischeri* (15)	n.d.	39.36 (±2.25)	61.88 (±6.05)	177.96 (±49.07)	n.d.	5	n.d.
	V. fischeri (30)	n.d.	31.88 (±5.91)	61.23 (±19.7)	125.83 (±62.22)	n.d.	5	n.d.
	P. tricornutum	n.d.	n.d.	2.98 (±19.14)	3.343 (±20.61)	5	25	n.d.
	I. galbana	28.62 (±40.62)	40.85 (±57.04)	62.54 (±86.96)	119.6 (±167.64)	25	50	1.6
	P. lividus	n.d.	n.d.	32.89 (±6.19)	36.14 (±6.16)	n.d.	5	n.d.
	A. lixula	19.86 (±1.77)	21.19 (±1.46)	23.15 (±1.06)	28.48 (±1.71)	5	25	4.2
IV	*V. fischeri* (15)	9.56 (±7.87)	37.30 (±36.19)	67.63 (±70.93)	130.57 (±149.13)	n.d.	5	n.d.
	V. fischeri (30)	n.d.	39.89 (±17.15)	67.36 (±36.01)	129.67 (±88.01)	n.d.	5	n.d.
	P. tricornutum	3.86E−43 (±2.17E−40)	7.43E−01	3.09E+ 03 (±4.35E+106)	n.d.	n.d.	5	n.d.
	I. galbana	n.d.	13.86 (±18.5)	n.d.	n.d.	n.d.	5	n.d.
	P. lividus	2.11 (±2.31)	4.61 (±6.52)	10.36 (±18.89)	33.22 (±82.24)	5	25	1.0
	A. lixula	4.6 (±0.83)	5.41 (±0.89)	6.75 (±0.98)	11.2 (±1.57)	5	25	1.0
V	*V. fischeri* (15)	15.877 (±2.91)	25.95 (±4.93)	42.46 (±12.59)	103.91 (±57.7)	5	25	5.2
	V. fischeri (30)	n.d.	24.47 (±1.8)	35.12 (±2.24)	82.05 (±15.72)	n.d.	5	n.d.
	P. tricornutum	n.d.	n.d.	n.d.	n.d.	50	75	n.d.
	I. galbana	n.d.	n.d.	n.d.	n.d.	5	25	n.d.
	P. lividus	4.26 (±0.21)	4.82 (±0.21)	5.71 (±0.24)	8.47 (±0.6)	n.d.	5	n.d.
	A. lixula	x	x	x	x	x	x	x
VI	*V. fischeri* (15)	20.42 (±3.86)	53.33 (±18.81)	n.d.	n.d.	5	25	10.6
	V. fischeri (30)	14.19 (±4.27)	32.42 (±14.82)	n.d.	n.d.	5	25	6.4
	P. tricornutum	n.d.	n.d.	5.78 (±23.45)	3.18 (±20.78)	n.d.	n.d.	n.d.
	I. galbana	44.32 (±85.24)	51.49 (±107.61)	66.1 (±156.53)	115.72 (±346.53)	25	50	2.1
	P. lividus	n.d.	28.85 (±23.92)	57.09 (±17.44)	101.02 (±109.22)	50	75	0.58
	A. lixula	21.93 (±7.24)	22.62 (±5.7)	23.59 (±3.45)	26.04 (±2.75)	n.d.	5	n.d.
VII	*V. fischeri* (15)	43.95 (±10.25)	70.83 (±48.2)	155.32 (±227.79)	n.d.	25	50	2.8
	V. fischeri (30)	39.74 (±25.52)	75.01 (±68.56)	122.7 (±139.71)	n.d.	n.d.	5	n.d.
	P. tricornutum	n.d.	38.32 (±22.89)	n.d.	n.d.	n.d.	n.d.	n.d.
	I. galbana	14.79 (±10.84)	23.85 (±20.29)	41.61 (±41.52)	103.68 (±129.75)	5	25	4.8
	P. lividus	n.d.	22.22 (±1.16)	25.51 (±1.07)	31.09 (±1.47)	5	25	4.4
	A. lixula	3.94 (±1.1)	4.51 (±0.54)	5.24 (±0.3)	6.76 (±2.39)	n.d.	5	n.d.
VIII	*V. fischeri* (15)	n.d.	47.34 (±12.87)	51.26 (±6.59)	n.d.	25	50	1.9
	V. fischeri (30)	15.53 (±5.54)	21.94 (±5.41)	33.18 (±7.73)	150.41 (±126.24)	5	25	4.4
	P. tricornutum	5.43 (±20.5)	4.35 (±17.07)	2.89 (±12.14)	0.93 (±4.63)	5	25	0.8
	I. galbana	5.46 (±14.46)	4.27 (±12.17)	2.66 (±8.63)	0.66 (±2.91)	n.d.	5	n.d.
	P. lividus	0.82 (±6.27)	1.11 (±7.07)	1.54 (±7.66)	2.71 (±6.96)	n.d.	5	n.d.
	A. lixula	4.86 (±0.33)	5.1 (±0.25)	5.43 (±1.1)	6.31 (±3.6)	n.d.	5	n.d.

Data not available; n.d., not determined.
EC_x values with their 95% confidence intervals (±CI), NOEC and LOEC are expressed as effluent percentage.

or stimulated (trophic effect). *Phaeodactylum tricornutum* suffered toxic effects at the highest dilutions, with growth inhibition of around 20% or higher relative to the control individuals (I, II, III, IV and VI) (Fig. 3). Thereafter, growth remained almost constant (effluents I, II, IV, VII) or increased significantly ($p<0.05$), in some cases exceeding that of control individuals (effluents V and VIII), which indicated possible eutrophication. *Isochrysis aff. galbana* showed variable responses at the highest dilutions, with growth ranging between 10% inhibition and 20% increase (Fig. 3). Thereafter, growth exhibited significant ($p<0.05$) detrimental effects (effluents II, III, VI and VII), or increased significantly (effluent VIII), leading to possible eutrophication. Growth of the macroalgae remained almost constant only when microalgae were exposed to effluents I and VII. The low variation in *P. tricornutum* growth, along with the effluent dilutions, prevented calculation of most of the EC values, so they were estimated from the fitted model. Student's *t* tests and Mann–Whitney tests performed to compare species showed significant differences ($p<0.05$) (Table 4). Furthermore, species were compared according to the characteristics of the response. Growth of *P. tricornutum* was inhibited at low percentages of effluent, but from a concentration of 5%, the effluent dilutions barely affected the organisms. On the contrary, growth of *I. galbana* varied significantly with increasing effluent concentrations. The latter species therefore appeared to be most suitable for explaining the influence of effluent concentrations on growth. Effluents III, VI and VII were found to be the most toxic to the microalgae, exhibiting the lowest EC_5, EC_{10}, EC_{20} and EC_{50} values, while effluent VIII was found to improve growth.

3.3. Sea urchin embryo development test

Development of both *P. lividus* and *A. lixula* was clearly related to the effluent dilutions: the percentage of abnormal larvae increased with increasing concentration of effluent, except for *P. lividus* exposed to effluent I. The sensitivity to the different dilutions of effluent enabled calculation of most of the EC values.

Table 4
P-values obtained from ANOVA, Mann–Whitney and Student's *t* analyses.

	All data	Bacteria	Microalgae	Sea urchin
ANOVA				
5%	0.107	0.000*	0.247	0.005*
25%	0.128	0.000*	0.000*	0.000*
50%	0.921	0.000*	0.000*	0.029*
75%	0.890	0.000*	0.000*	0.002*
100%	0.732	0.000*	0.000*	0.004*
Mann–Whitney		0.291	0.002**	0.085
Student's *t*		0.245	0.024**	0.148

ANOVA was conducted to determine the variability of results at the different dilutions, using the whole set of results (all data) and results of each toxicity test (bacteria, microalgae and sea urchin). The preferred dilution is highlighted in gray color. Mann–Whitney and Student's *t*-test were performed to identify significant differences between exposure times (bacteria test) and species (microalga and sea urchin tests).

* Significant differences between LBMFFs.
** Significant differences between pair of species.

Comparison of the ECs of both species did not reveal any significant differences ($p < 0.05$) (Table 4). Nonetheless, *A. lixula* showed lower values in 56.5% of the cases, particularly in parameters EC_{20} and EC_{50}, which indicated a slightly higher sensitivity than that shown by *P. lividus*. The lowest EC values were observed with effluents I, IV and VIII, and in the case of *A. lixula*, also with effluent VII. The highest EC values corresponded to effluents II and VI.

3.4. Comparison of the sensitivity of the toxicity tests

Sea urchins proved to be the most sensitive organisms when exposed to discharges from fish farms I, V and VII, regardless of the EC considered. Effluents II, III, IV and VIII had the greatest effects on sea urchins and microalgae, depending on the EC considered. Effluent VI showed similar degrees of toxicity to all species.

The 5% dilution was found to be the best at explaining the variability ($p = 0.107$) among the toxicity of the LBMFF effluents when the ANOVA analysis included the whole set of data, i.e. all bioassays (Table 4). Nevertheless, when ANOVA was conducted separately for each bioassay, the 5% dilution was the only dilution that did not show significant differences among fish farms according to the microalgal bioassay. Therefore, considering the degree of significance explained by each bioassay, the 25% dilution best explained the differences among the different farms. Kernel density distribution of inhibition (Fig. 5) demonstrated that the 30 min *V. fischeri* assay and the embryo development test with *A. lixula* were the most sensitive bioassays for effluent pollutants when species were exposed to 25% dilution of the effluents. As regards the microalgae tests, if the highest percentages of growth inhibition had been taken into account, *P. tricornutum* would have been considered the most sensitive, but increasing effluent concentrations did not affect growth of this microalgae. On the contrary, growth of *I. galbana* decreased with increasing effluent concentrations in most cases, so that this species better explained changes in growth provoked by changes in effluent concentrations.

3.5. Potential ecotoxic effects

The small number of data obtained using the original formula with NOEC and LOEC values limited and reduced estimation of the PEEP values. Therefore, EC_{10}, EC_{20} were used to calculate the PEEP index. The toxic units (TU) obtained in each bioassay for each farm effluent, and the toxic print, the effluent flow, the toxic loading and the PEEP index values obtained in each LBMFF are shown in Table 5. In most cases, the TU for EC_{10} and EC_{20} were very similar. The sea urchin and microalgae tests were the main contributors to the toxic print. The highest toxic prints corresponded to fish farms IV (187.1 TU) and VIII (162.8 TU), with *P. tricornutum* and *P. lividus* providing most of the TU (134.5 and 90.1, respectively). However, the TU values obtained with *P. tricornutum* were not reliable because they were extrapolated from the fitted model.

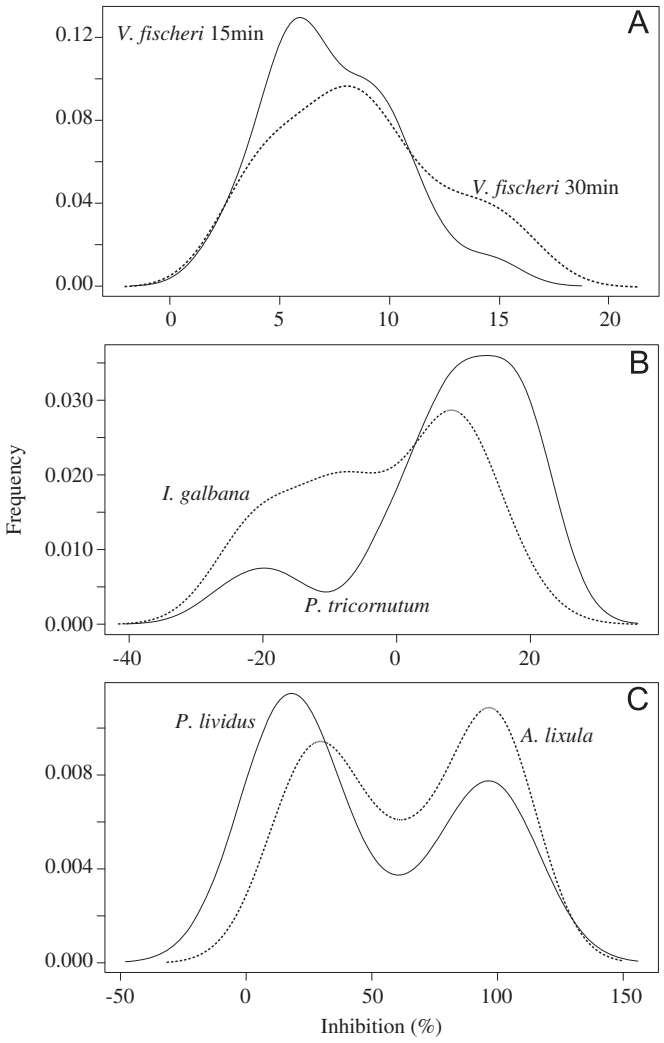


Fig. 5. Frequency distribution of the results obtained with the 25% dilution of the effluent, in the bioassays with bacteria (A), microalgae (B) and sea urchins (C), according to the Kernel estimation with Gaussian basis function.

4. Discussion

As LBMFFs are usually located on highly exposed coasts, discharged waste is therefore rapidly removed or diluted at a close distance from the output source. The dispersion capacity of the environment is difficult to assess since the areas that are directly affected are not usually very extensive. Several studies have reported that effluents from fish farms affect the environmental conditions of the water column and the sediment around the cages, especially in the intensive culture of carnivorous fish, which require large amounts of manufactured feed (Holmer, 2010). It has been reported that at large spatial and temporal scales, the effects of fish farms on water quality are not relevant

Table 5
Toxic units, toxic print, effluent flow (Q), toxic load and PEEP index values obtained from the results of the different bioassays for each fish farm effluent, according to the EC_{10}/EC_{20} values.

		Toxic Units						Toxic print	Q (m^3h^{-1})	Toxic load	PEEP index
		Decomposer		Primary producer		Primary consumer					
		V. fischeri, 15 min	*V. fischeri*, 30 min	*P. tricornutum*	*I. galbana*	*P. lividus*	*A. lixula*				
LBMFF	I	1.4/*x*	1.3/0.6	*x*/*x*	1.4/1.0	20.0/20.0	20.7/16.0	37.4/25.1	45,014	1,681,628/1130,384	6.2/6.1
	II	*x*/2.0	*x*/2.0	*x*/9.9	1.1/0.9	*x*/2.7	4.2/4.0	1.8/21.4	5842	10,331/125,186	4.0/5.1
	III	2.5/1.6	3.1/1.6	*x*/33.6	2.4/1.6	*x*/3.0	4.7/4.3	8.6/45.8	6160	52,748/281,915	4.7/5.5
	IV	2.7/1.5	2.5/1.5	134.5/*x*	7.2/*x*	21.7/9.7	18.5/14.8	187.1/18.3	23,311	4,361,534/426,296	6.6/5.6
	V	3.8/2.4	4.1/2.8	*x*/*x*	*x*/*x*	20.7/17.5	–/–	17.2/13.6	6963	119,860/94,910	5.1/5.0
	VI	1.9/*x*	3.1/*x*	*x*/17.3	1.9/1.5	3.5/1.8	4.4/4.2	12.3/16.5	872	10,751/14,426	4.0/4.2
	VII	1.4/0.6	1.3/0.8	2.6/*x*	4.2/2.4	4.5/3.9	22.2/19.1	36.2/22.4	5690	206,112/127,400	5.3/5.1
	VIII	2.1/2.0	4.5/3.0	22.9/34.6	23.4/37.6	90.1/64.9	19.6/18.4	162.8/160.5	1020	166,130/163,819	5.2/5.2
	Mean							48.5/40.5	11,859	826,137/295,542	5.2/5.2

x, toxicity value not detected in the bioassay; –, data not available.

(Pitta et al., 2006), because the waste is rapidly diluted. Although the release of nutrients from fish farms has traditionally been monitored by measuring physical or chemical variables, this approach provides little information about environmental quality if detailed spatio-temporal and expensive sampling is not considered. In order to improve the methods used for aquaculture impact monitoring, a biological study of the potential ecotoxicological effects of LBMFFs was carried out, for the first time, by the application of a selected battery of biotests.

There is no "most sensitive species" because organisms display different degrees of sensitivity towards substances or their mixtures (Bakopoulou et al., 2011; Van der Grinten et al., 2010). However, in this and previous studies (Carballeira et al., 2010), a sea urchin bioassay in which skeletal deformities are measured has been found to be an accurate method of determining the potential toxic effects of LBMFF effluents. The toxic effects of fish farm sediments have previously been observed in clams and sea urchins, directly underneath cages (Kullman et al., 2007; Marin et al., 2007). During the maximum production period (summer) a toxic response was also registered by the sea urchin bioassay, up to 125 m from fish cages (Marin et al., 2007).

The Microtox® bacterial bioluminescence test, used as part of a battery of bioassays, has long been considered to be extremely sensitive (Park and Choi, 2008). Moreover, this test has been successfully developed to test the toxicity of antibiotics (Backhaus and Grimme, 1999; Isidori et al., 2005; Lalumera et al., 2004), and organic chemicals (Hernando et al., 2007) used in marine aquaculture. However, the present results showed that the Microtox® test was the least sensitive bioassay, despite the high precision of data (small standard deviation). These results are consistent with those of other toxicity studies (Fernández-Alba et al., 2002; Macken et al., 2008; Mankiewicz-Boczek et al., 2008; Schipper et al., 2010). *V. fischeri* has been found to present low sensitivity when exposed to compounds as the antibiotics oxytetracycline (Isidori et al., 2005; Lalumera et al.; 2004; Park and Choi, 2008), amoxicillin (Park and Choi, 2008) and ampicillin (Backhaus and Grimme, 1999; Chi, 2009; Park and Choi, 2008), and metals (Petala et al., 2005). The aforementioned antibiotics are commonly used to treat diseases in diverse farmed fish species (Lalumera et al., 2004), while several metals have been found to be trace components of fish feed (Ikem and Egilla, 2008) and main components of widespread biocides (Braithwaite and McEvoy, 2004). A decrease in the bacteria sensitivity has also been reported when increasing the complexity of the composition of the aqueous solution that was tested, by including different organic and inorganic compounds as insecticides, herbicides or pharmaceuticals (Codina et al., 1993; Munkittrick et al., 1991). Substances with delayed toxicity may show low or no toxicity in the standardized short term bioluminescence bioassay (30 min); however, long-term bioassays may help to overcome these limitations (Backhaus and Grimme, 1999; Froehner et al., 2000; Wang et al., 2009). The increase of the exposure time in the Microtox® test may lead to a greater sensitivity, as it was reported for oxytetracycline (Park and Choi, 2008), for formaldehyde (Chou and Que Hee, 1992) and for several metals (Petala et al., 2005) and wastewaters enriched with metals (Choi and Meier, 2001).

Microalgae have been found to be sensitive to a variety of contaminants associated with aquaculture activities. Muñoz et al. (2010) reviewed the toxicity of aquaculture micropollutants and found that microalgae were the most sensitive organisms in a battery of bioassays that included fish and crustaceans. Similarly, Fernández-Alba et al. (2002) found that microalgae were the most sensitive organisms against a series of antifoulants in a battery of bioassays that included bacteria and crustacean. Källqvist and Svenson (2003) reported high sensitivity of microalgae to ammonia. This (i) supported our decision of selecting microalgae as test organisms for the assessment of aquaculture effluents, and (ii) agreed with our results, which exhibited high sensitivity of microalgae to LBMFF effluents, which may contain a mixture of some of the contaminants evaluated by the afore mentioned authors. The results obtained with microalgae in the present work showed two types of responses with particular gradients: growth inhibition (toxic response) and growth stimulation (trophic response). In general, *P. tricornutum* showed a flat response in relation to discharge dilution, whereas *I. galbana* showed a toxic-trophic gradient in relation to dilution. Growth responses derived from the exposure to the LBMFF effluents were likely to vary according to specific contaminant mixtures, resulting from the balance of contaminants and nutrients (specially N) contained in the effluents. Algae are known to be saturated of nutrients (with the maximum growth at a specific temperature) when exposed to natural seawater from Galicia coast (Villares and Carballeira, 2004, 2006). Because this test was performed using Galician natural seawater, microalgae nutrient saturation was probably 100% regardless the dilution. Thus, at high dilutions (e.g. 5%, 10%), the 100% nutrient saturation and the low toxicity of the highly diluted effluents might have led to growth stimulation. However, at lower effluent dilutions, nutrient saturation might have remained at 100%, but toxicity increased leading to growth inhibition. In this manner the lower the dilution of the effluent, the greater the toxicity.

Since urban and industrial sewage waters contain large amounts of organic matter, disinfectants and antibiotics, the characteristics of aquaculture effluents and potentially additive

effects may be compared with those of the aforementioned sewage waters. Bacteria, microalgae and invertebrates are the organisms most commonly used to monitor the toxicity of municipal wastewaters (Moreno-Garrido, 2008; Radjenovic et al., 2009). In general, *V. fischeri* has been found to be more sensitive than algae in tests of the toxicity of sewage waters of different origin (Pandard et al., 2006). In most studies with industrial waste, the Microtox® test has been reported to be more sensitive to contamination than tests with microalgae and small crustaceans (Kungolos et al., 2009; Macken et al., 2008; Roig et al., 2011). Nevertheless, it should be borne in mind that the toxicity of numerous chemicals (metals, hydrocarbons, biocides and pesticides) decreases with increasing salinity (Hall and Anderson, 1995; Latala et al., 2010) and that organisms from the same trophic level may show different responses.

The validity of the proposed method of assessing the impact of LBMFFs on the environment, which included three tests with species from different trophic levels, has been demonstrated. Nevertheless we believe that the efficacy of the recommended test battery would be improved by using specific contaminants present in the fish farm effluents (i.e. disinfectants, antibiotics, ammonia, etc.—both isolated and in mixtures) and by studying the interactions between these substances and environmental factors (Nendza, 2002). In fact, we have recently used the bioassays described in this work with several of these products (Carballeira et al., Submitted-a; De Orte et al., 2009; Viana et al., Submitted), in order to characterize the response of the different species to particular pollutants and mixtures of pollutants. High concentrations of ammonia may have explained the results of the sea urchin test, as *P. lividus* has been shown to be very sensitive to this substance (Arizzi Novelli et al., 2003; Carballeira et al., Submitted-a; Garmendia et al., 2009). However, in this bioassay there was no clear correlation between the toxicity of the effluent and levels of ammonia. However, this is perhaps not surprising because LBMFF effluents are complex mixtures of different types of pollutants, the effects of which may interfere with each other.

ANOVA (Dunnett test) has been widely used to calculate NOEC and LOEC values from the toxic effect data. However, the reliability and accuracy of these toxicological parameters strongly depend on the concentrations tested and the number of replicates used (Chapman et al., 1996; Isidori et al., 2005). An alternative procedure is the estimation of EC_x values by regression analysis. The EC_{50} is regarded as the most robust endpoint in toxicological studies. However, the LBMFF produced effluents of generally low toxicity, which prevents calculation of EC_{50} in many cases. Moreover, the EC_{50} may not be considered a protective criteria from an ecological perspective. The NOEC indicated imprecise inhibition, which varied between 5% and 30% with respect to the control. The EC_{10}/NOEC ratios from different tests have been found to be higher than one (around 1.3) (Isnard et al., 2001; Shieh et al., 2001), whereas the present results showed that most of the EC_{10}/NOEC values (when able to be calculated) varied widely, between 1 and 15. Furthermore, the EC_{10} has the disadvantage of being more imprecise because the accuracy of the regression models decreases in the low effect zone (Isnard et al., 2001; Shieh et al., 2001; Sbrilli et al., 2005). In this work, the PEEP index was calculated by using both EC_{10} and EC_{20} (Table 5). Broadly, the classification of the potential toxicity of the farms using the EC_{10} and using the EC_{20} were similar. They agreed on the higher toxic potential of farms I and IV, and in the lower toxic potential of farms II and VI. Moreover, the confidence intervals were similar for both EC_{10} and EC_{20}. Nevertheless, it has been previously reported in various ecotoxicity guidelines and research works that EC_{20} is often more statistically robust and precise than EC_{10} (Environment Agency of England and Wales, 2006; OECD, 2007; Boeije et al., 2006), because the lower the effect zone, the larger the confidence intervals appeared to be (Isnard et al., 2001; Shieh et al., 2001; Sbrilli et al., 2005). Furthermore, while several drawbacks on the use of EC_{10} were found in literature (in comparison with NOEC or EC_{20}), we could not find objections to the use of EC_{20}. Therefore, EC_{20} appeared to be more realistic and statistically more robust than EC_{10}, and represented a permissible degree of inhibition. In consequence, EC_{20} was recommended for calculation of the PEEP index.

The TU derived from EC_{20} confirmed the higher sensitivity of the sea urchin in assessing the effluent toxicity of the LBMFFs. The toxic print correctly integrated the potential toxicity of the effluent, particularly from farms III and VIII. However, the highest PEEP index values corresponded to farms I (6.05) and IV (5.63), because of the higher flow rates at these sites. The PEEP values were therefore similar to the highest values (ranging between 0.78 and 7.6) obtained for industries that discharge potentially more toxic substances (i.e. inorganic and organic chemical production plants, pulp and paper mills and municipal incinerators) (Blaise and Férard, 2005). The toxic loading is considered to be a valuable tool for inclusion within Ecological Risk Assessment because it combines the toxicity and the volume of waste discharged. However, the dispersive capacity of the receiving environment must also be taken into account. The dispersive capacity of a coastal environment is difficult to estimate because it depends on the interaction between numerous factors such as the direction of the current and velocity at different depths, the intensity and frequency of the waves, the topography and exposure to prevailing winds. One possible way of evaluating waste dispersion is to determine the $\delta^{15}N$ of macroalgae at the area of influence of the LBMFFs, since this has already been reported to be a valuable descriptor of the dispersive capacity of the environment (Carballeira et al., Submitted-b).

The bioassay results obtained with the 25% effluent dilution, which explained the highest variability between LBMFFs, were graphically summarized using a triaxial diagram. This representation provided a global view of the results, making the differences amongst farm effluent toxicities more evident. The endpoints for the most sensitive species (*V. fischeri* 30 min, *I. galbana* and *A. lixula*) were considered (Fig. 6). Average values from all LBMFFs were represented as a dashed line together with the standard deviation. The values obtained in the *P. lividus* bioassay were used in the sea urchin axis in the triaxial diagram for fish farm V because of the lack of data for *A. lixula* and because there were no significant differences between the sensitivity of larvae of either species, in any case. This diagram provided an easy and integrative interpretation of the toxicity of each farm effluent. This integrative method revealed the higher sensitivity of the sea urchin bioassay and showed that effluents from fish farms II and VI were the least toxic, while the effluent from farm VIII was the most toxic, probably because of the higher concentration of contaminants originating from several water depuration treatments used on this farm. The graphical representation agreed with the classification based on the PEEP index on considering effluents II and VI the less potentially toxic. However, a lack of agreement was found when determining the most potentially toxic effluent, since the graphical representation did not take into account the effluent flow. The determination of the concentration with which differences amongst farms are greater (25% dilution in this particular case) can be used as a preliminary approach to the assessment of effluent toxicity and can included in LBMFF monitoring plans for effluent screening.

5. Conclusions

The present study is the first research work aiming the evaluation of the toxicity of effluents from LBMFFs. Specifically,

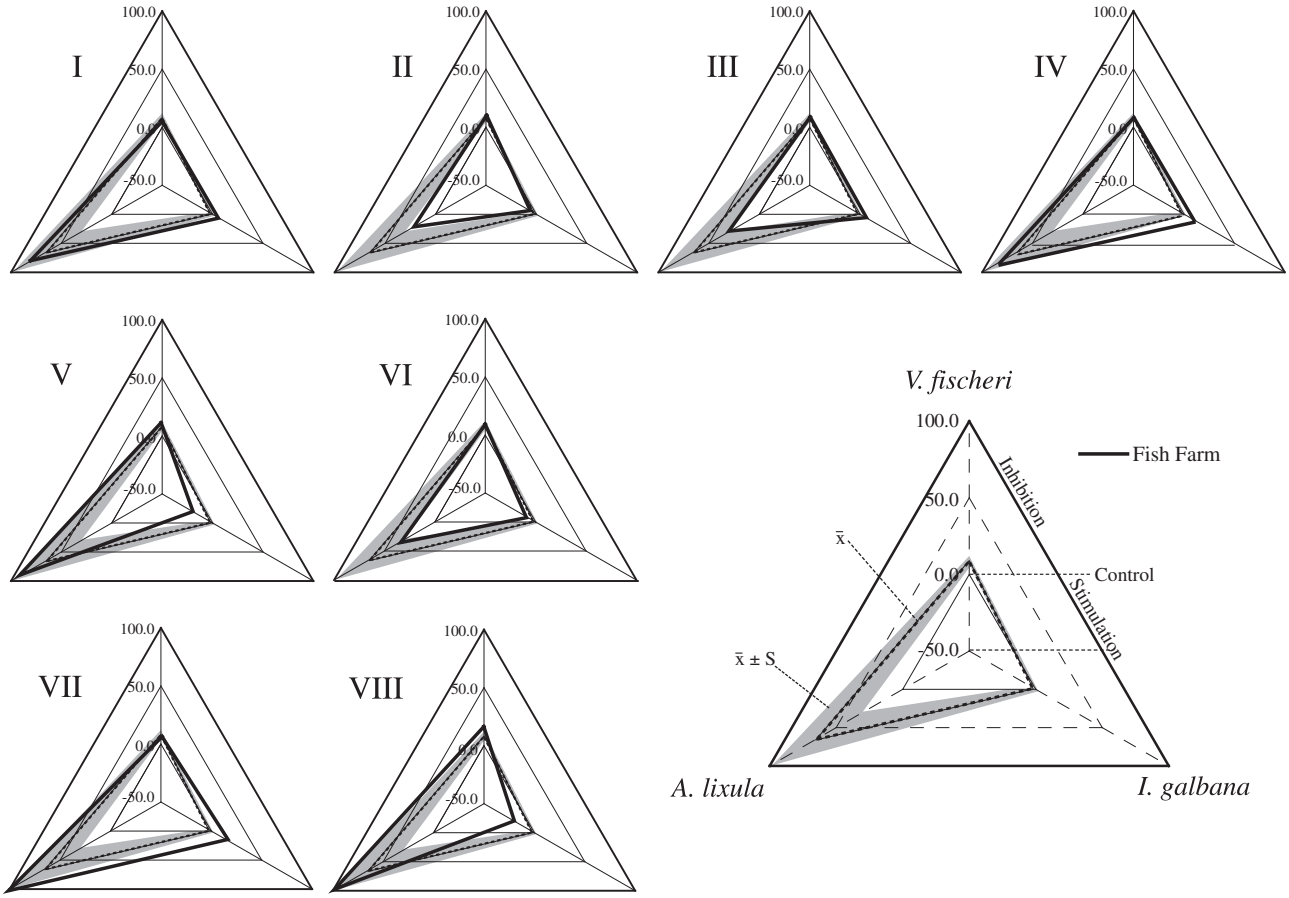


Fig. 6. Graphical integration of the results from of the most sensitive bioassays in this study. Each fish farm is represented by a solid line, while the average values are represented by dashed lines. Results are expressed as inhibition percentage, relative to the control. Gray area represents mean values together with the standard deviation. $\bar{x}$: mean values, S: standard deviation.

the effluents of 8 LBMFFs (20.5% of the total number of LBMFFs in Spain) were assessed. Although official data about the use of chemicals in the farms were not available, they were expected not to present significant disparities amongst each other nor in comparison with other LBMFFs located in different regions of the world, because all LBMFFs have the same chemical requirement (e.g. facilities cleaning and disinfection, and diseases prevention and treatment), use similar fish feed and they all generate the same types of residues (feces, urine, rests of feed, dissolved chemical products). Nevertheless, differences can be found in the concrete chemicals used and in the effluent flow. In this sense, the fact of studying 8 LBMFFs that grow different fish species, and have different level of production and different effluent flow, covered a wide spectrum of farms, providing validity and representativeness to the methodology used. Nevertheless, it was not possible to compare the effectiveness of this methodology with others nor with studies conducted in other regions of the world because, to date, similar works have not been performed.

A battery of standardized toxicity tests including at least one species of bacteria (long exposure *V. fischeri*), one species of microalgae (*I. galbana*) and one invertebrate species (*A. lixula*) is recommended for assessment of the environmental impact of LBMFF effluents. This battery of tests enables cost-effective ecotoxicological monitoring by the detection and characterization of the toxic effects of such effluents.

EC_{20} values were found to be more useful than EC_{50} values for comparing the toxic effects of LBMFFs. The PEEP index calculated from the EC_{20} values distinguished the level of toxicity of LBMFF effluents, which generally have low toxic potential. This index is easy to use and interpret and is a cost-effective tool for determining the potential toxic loading of effluent, and so may improve environmental risk assessment studies. The data described may aid in future management decisions for land-based aquaculture and contribute to new efforts being made within programs such as the WFD (Water Framework Directive).

Acknowledgments

The present study was partly financed by the Spanish Government's National Plan for Marine Culture (JACUMAR, 2008): "Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)". Carlos Carballeira is grateful to the University of Cadiz Predoctoral Fellowships Programme (Spain). Manoela De Orte thanks the Erasmus Mundus scholarship provided by the European Union.

References

Aguirre-Martínez, G.V., Carballeira, C., Martín-Díaz, M.L., DelValls, T.A., 2011. Optimization of fertilization and larval development toxicity tests using two marine sea urchin species. Study of salinity influence. In: SETAC (Ed.), Ecosystem Protection in a Sustainable World: A Challenge for Science and Regulation. SETAC Milan, 164–165.

APROMAR (Asociación Empresarial de Productores de Cultivos Marinos de España), 2011. La acuicultura marina en España. 〈www.apromar.es/Informes/〉.

Arizzi Novelli, A., Picone, M., Losso, C., Volpi Ghirardini, A.M., 2003. Ammonia as confounding factor in toxicity tests with the sea urchin *Paracentrotus lividus* (Lmk). Toxicol. Environ. Chem. 85, 183–191.

Azur Environmental, 1998. Microtox® Test Manual. In: Environmental, A. (Ed.)., Strategic Diagnostics Inc., Carlsbad.

Backhaus, T., Grimme, L.H., 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. Chemosphere 38, 3291–3301.

Bakopoulou, S., Emmanouil, C., Kungolos, A., 2011. Assessment of wastewater effluent quality in Thessaly region, Greece, for determining its irrigation reuse potential. Ecotoxicol. Environ. Saf. 74, 188–194.

Beiras, R., Vázquez, E., Bellas, J., Lorenzo, J.I., Fernández, N., Macho, G., Mariño, J.C., Casas, L., 2001. Sea-urchin embryo bioassay for *in situ* evaluation of the biological quality of coastal seawater. Estuar. Coast. Shelf Sci. 52, 29–32.

Blaise, C., Férard, J.-F., 2005. Effluent assessment with the PEEP (potential ecotoxic effects probe) index. In: Férard, B.A. (Ed.), Small-Scale Freshwater Toxicity Investigations. , Springer, Netherlands, pp. 69–87.

Boeije, G.M., Cano, M.L., Marshall, S.J., Belanger, S.E., Van Compernolle, R., Dorn, P.B., Gümbel, H., Toy, R., Wind, T., 2006. Ecotoxicity quantitative structure–activity relationships for alcohol ethoxylate mixtures based on substance-specific toxicity predictions. Ecotoxicol. Environ. Saf. 64, 75–84.

Borja, Á., 2002. Los impactos ambientales de la acuicultura y la sostenibilidad de esta actividad. Bol. Inst. Esp. Oceanogr. 18, 41–49.

Braithwaite, R.A., McEvoy, L.A., 2004. Marine biofouling on fish farms and its remediation. Adv. Mar. Biol. Academic Press 47, 215–252.

Carballeira, C., De Orte, M., Viana, I.G., Del Valls, T.A., Assessing the ecotoxicity of chemicals compounds associated to land-based marine fish farms: sea urchin embryo bioassay with *Paracentrotus lividus* and *Arbacia lixula*. Arch. Environ. Contam. Toxicol., submitted-a for publication.

Carballeira, C., Martín-Diaz, M.L., Del Valls, T.A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos con embriones de erizo. In: Rey Méndez, M., Fernández Casal, J., Izquierdo Rodríguez, M., Guerra Díaz, A. (Eds.), XII Foro dos recursos mariños e da acuicultura das rías galegas., USC, O Grove, pp. 177–184.

Carballeira, C., Viana, I., Carballeira, A., $\delta^{15}N$ in macroalgae as an indicator of the potential impact of waste disposal from land based fish farms. Mar. Pollut. Bull., submitted-b for publication.

Cesar, A., Marín, A., Marín-Guirao, L., Vita, I., 2004. Amphipod and sea urchin tests to assess the toxicity of Mediterranean sediments: the case of Portmán Bay. Sci. Mar. 68, 205–213.

Chapman, P.M., Caldwell, R.S., Chapman, P.F., 1996. A warning: NOECs are inappropriate for regulatory use. Environ. Toxicol. Chem. 15, 77–79.

Chi, S.C.D., 2009. Eco-toxicity of antibiotics on aquatic organism. Master Thesis. Hong Kong University, Hong Kong, p. 78.

Choi, K., Meier, P.G., 2001. Toxicity evaluation of metal plating wastewater employing the microtox assay: a comparison with cladocerans and fish. Environ. Toxicol. 16, 136–141.

Chou, C.C., Que Hee, S.S., 1992. Microtox EC50 values for drinking water by-products produced by ozonolysis. Ecotoxicol. Environ. Saf. 23, 355–363.

Codina, J.C., Pérez-García, A., Romero, P., Vicente, A., 1993. A comparison of microbial bioassays for the detection of metal toxicity. Arch. Environ. Contam. Toxicol. 25, 250–254.

Coelho, S., Oliveira, R., Pereira, S., Musso, C., Domingues, I., Bhujel, R.C., Soares, A.M.V.M., Nogueira, A.J.A., 2011. Assessing lethal and sub-lethal effects of trichlorfon on different trophic levels. Aquat. Toxicol. 103, 191–198.

De Orte, M., Carballeira, C., Carballeira, A., 2009. Desarrollo de un bioensayo miniaturizado de microalgas para evaluar la ecotoxicidad de los vertidos y los productos utilizados en piscifactorías marinas instaladas en tierra, European Joint Master in Water and Coastal Management. University of Cadiz, Cadiz p. 54.

Dean, R.J., Shimmield, T.M., Black, K.D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: an extensive survey. Environ. Pollut. 145, 84–95.

DelValls, T.A., 2007. Diseño y aplicación de modelos integrados de evaluación de la contaminación y sus efectos sobre los sistemas marinos y litorales y la salud humana. Ministerio de la Presidencia. Secretaría General Técnica. Centro de Publicaciones, Madrid.

Environment Agency of England and Wales, 2006. Integrated pollution prevention & control (IPPC). Guidance on the use of direct toxicity assessment in PPC impact assessments. 〈www.environment-agency.gov.uk/business/sectors/117135.aspx〉.

Fabregas, J., Abalde, J., Herrero, C., Cabezas, B., Veiga, M., 1984. Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. Aquaculture 42, 207–215.

FAO, 2010. In: Department, F.A.O. (Ed.), The state of world fisheries and aquaculture. , Food and Agricultural Organization of the United Nations, Rome, pp. 218.

Fernandes, T.F., Miller, K.L., Read, P.A., 2000. Monitoring and regulation of marine aquaculture in Europe. J. Appl. Ichthyol. 16, 138–143.

Fernández, N., Beiras, R., 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10, 263–271.

Fernández-Alba, A.R., Hernando, M.D., Piedra, L., Chisti, Y., 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. Anal. Chim. Acta 456, 303–312.

Froehner, K., Backhaus, T., Grimme, L.H., 2000. Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. Chemosphere 40, 821–828.

Garmendia, J.M., Menchaca, I., Belzunce, M.J., Revilla, M., 2009. Protocolo del test de toxicidad de sedimentos marinos con larvas del erizo de mar *Paracentrotus lividus* (Lamarck, 1816), Revista de Investigación Marina, first ed. AZTI, Pasaia, p. 25.

GESAMP, 1996. Monitoring the Ecological Effects of Coastal Aquaculture Wastes. FAO, Rome.

Hall, L.J., Anderson, R., 1995. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. Crit. Rev. Toxicol. 25, 281–346.

Hansen, P.K., Ervik, A., Schaanning, M., Johannessen, P., Aure, J., Jahnsen, T., Stigebrandt, A., 2001. Regulating the local environmental impact of intensive, marine fish farming: II. The monitoring programme of the MOM system (Modelling-Ongrowing fish farms-Monitoring). Aquaculture 194, 75–92.

Hernando, M.D., De Vettori, S., Martínez Bueno, M.J., Fernández-Alba, A.R., 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere 68, 724–730.

Hirmann, D., Loibner, A.P., Braun, R., Szolar, O.H.J., 2007. Applicability of the bioluminescence inhibition test in the 96-well microplate format for PAH-solutions and elutriates of PAH-contaminated soils. Chemosphere 67, 1236–1242.

Holmer, M., 2010. Environmental issues of fish farming in offshore waters: perspectives, concerns and research needs. Aquacult. Environ. Interact. 1, 57–70.

Ikem, A., Egilla, J., 2008. Trace element content of fish feed and bluegill sunfish (*Lepomis macrochirus*) from aquaculture and wild source in Missouri. Food Chem. 110, 301–309.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci. Total Environ. 346, 87–98.

Isnard, P., Flammarion, P., Roman, G., Babut, M., Bastien, P., Bintein, S., Esserméant, L., Férard, J.F., Gallotti-Schmitt, S., Saouter, E., Saroli, M., Thiébaud, H., Tomassone, R., Vindimian, E., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere 45, 659–669.

ISO 10253, 2006. Water quality—marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*, 22.

Jiangning, C., Hongxia, Y., Ying, L., Wei, J., Jie, J., Junfeng, Z., Zichun, H., 2004. Ecotoxicological evaluation of 4-aminobiphenyl using a test battery. Ecotoxicol. Environ. Saf. 58, 104–109.

Källqvist, T., Svenson, A., 2003. Assessment of ammonia toxicity in tests with the microalga, *Nephroselmis pyriformis*, Chlorophyta. Water Res. 37, 477–484.

Kullman, M.A., Podemski, C.L., Kidd, K.A., 2007. A sediment bioassay to assess the effects of aquaculture waste on growth, reproduction, and survival of *Sphaerium simile* (Say) (Bivalvia: *Sphaeriidae*). Aquaculture 266, 144–152.

Kungolos, A., Emmanouil, C., Tsiridis, V., Tsiropoulos, N., 2009. Evaluation of toxic and interactive toxic effects of three agrochemicals and copper using a battery of microbiotests. Sci. Total Environ. 407, 4610–4615.

Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., Fanelli, R., 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54, 661–668.

Latala, A., Nedzi, M., Stepnowski, P., 2010. Toxicity of imidazolium ionic liquids towards algae. Influence of salinity variations. Green Chem. 12, 60–64.

Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquat. Toxicol. 58, 27–41.

Macken, A., Giltrap, M., Foley, B., McGovern, E., McHugh, B., Davoren, M., 2008. A model compound study: the ecotoxicological evaluation of five organic contaminants employing a battery of marine bioassays. Environ. Pollut. 153, 627–637.

Mankiewicz-Boczek, J., Nalecz-Jawecki, G., Drobniewska, A., Kaza, M., Sumorok, B., Izydorczyk, K., Zalewski, M., Sawicki, J., 2008. Application of a microbiotests battery for complete toxicity assessment of rivers. Ecotoxicol. Environ. Saf. 71, 830–836.

Marin, A., Montoya, S., Vita, R., Marín-Guirao, L., Lloret, J., Aguado, F., 2007. Utility of sea urchin embryo-larval bioassays for assessing the environmental impact of marine fishcage farming. Aquaculture 271, 286–297.

Moreno-Garrido, I., 2008. Microalgae immobilization: current techniques and uses. Bioresour. Technol. 99, 3949–3964.

Munkittrick, K.R., Power, E.A., Sergy, G.A., 1991. The relative sensitivity of microtox®, daphnid, rainbow trout, and fathead minnow acute lethality tests. Environ. Toxicol. Water Qual. 6, 35–62.

Muñoz, I., Martínez Bueno, M.J., Agüera, A., Fernández-Alba, A.R., 2010. Environmental and human health risk assessment of organic micro-pollutants occurring in a Spanish marine fish farm. Environ. Pollut. 158, 1809–1816.

Nendza, M., 2002. Inventory of marine biotest methods for the evaluation of dredged material and sediments. Chemosphere 48, 865–883.

OECD, 1998. Report of the OCDE Workshop on statistical analysis of aquatic toxicity data. In: Development, OCDE (Ed.), Series on Testing and Assessment, pp. 133.

OECD (Organisation for Economic Co-operation and Development), 2007. OECD Guidelines for the Testing of Chemicals. Sediment–Water Lumbriculus Toxicity Test Using Spiked Sediment. 〈www.oecd.org/department/0,3355,en_2649_34377_1_1_1_1_1,00.html〉.

Pandard, P., Devillers, J., Charissou, A.M., Poulsen, V., Jourdain, M.J., Ferard, J.F., Grand, C., Bispo, A., 2006. Selecting a battery of bioassays for ecotoxicological characterization of wastes. Sci. Total Environ. 363, 114–125.

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17, 526–538.

Pavlic, Z., Stjepanovic, B., Horvatic, J., Persic, V., Puntaric, D., Culig, J., 2006. Comparative sensitivity of green algae to herbicides using Erlenmeyer flask and microplate growth-inhibition assays. Bull. Environ. Contam. Toxicol. 76, 883–890.

Pitta, P., Apostolaki, E., Tsagaraki, T., Tsapakis, M., Karakassis, I., 2006. Fish farming effects on chemical and microbial variables of the water column: a spatio-temporal study along the Mediterranean sea. Hydrobiologia 563, 99–108.

Petala, M., Tsiridis, V., Kyriazis, S., Samaras, P., Kungolos, A., Sakellaropoulos, G.P., 2005. Evaluation of toxic response of heavy metals and organic pollutants using Microtox acute toxicity test. In: 19th International Conferende on Environmental Science and Technology, Rhodes Island, Greece, pp. A-1200–A-1205.

Radjenovic, J., Petrovic, M., Barceló, D., 2009. Complementary mass spectrometry and bioassays for evaluating pharmaceutical-transformation products in treatment of drinking water and wastewater. Trends Anal. Chem. 28, 562–580.

R Development Core Team, R.D.C, 2008. R: a language and environment for statistical computing. Vienna Austria R Foundation for Statistical Computing 1, 7.

Read, P., Fernandes, T., 2003. Management of environmental impacts of marine aquaculture in Europe. Aquaculture 226, 139–163.

Rey-Asensio, A., Carballeira, C., Viana, I.G., Carballeira, A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioacumulación de microcontaminantes. In: Rey-Méndez, M., Lodeiros, C., Fernández Casal, J., Guerra, A. (Eds.), Foro dos Recursos mariños e da Acuicultura das Rías galegas XIII. , USC, O Grove, pp. 201–218.

Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., DeMarini, D.M., 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. Mutat. Res. 636, 178–242.

Ritz, C., Streibig, J.C., 2005. Bioassay analysis using R. J. Stat. Softw., 12.

Roig, N., Nadal, M., Sierra, J., Ginebreda, A., Schuhmacher, M., Domingo, J.L., 2011. Novel approach for assessing heavy metal pollution and ecotoxicological status of rivers by means of passive sampling methods. Environ. Int. 37, 671–677.

Roque D'Orbcastel, E., Sauzade, D., Ravoux, G., Coves, D., 2004. Methodological guide for the elaboration of creation of authorization classified installations for the environment protection (CIEP) in marine fish culture for the Corsica area. IFREMER, Brest, 370.

Sarà, G., 2007. A meta-analysis on the ecological effects of aquaculture on the water column: dissolved nutrients. Mar. Environ. Res. 63, 390–408.

Satoh, A., Vudikaria, L.Q., Kurano, N., Miyachi, S., 2005. Evaluation of the sensitivity of marine microalgal strains to the heavy metals, Cu, As, Sb, Pb and Cd. Environ. Int. 31, 713–722.

Sbrilli, G., Bimbi, B., Cioni, F., Pagliai, L., Luchi, F., Lanciotti, E., 2005. Surface and ground waters characterization in Tuscany (Italy) by using algal bioassay and pesticide determinations: comparative evaluation of the results and hazard assessment of the pesticides impact on primary productivity. Chemosphere 58, 571–578.

Schipper, C.A., Rietjens, I.M.C.M., Burgess, R.M., Murk, A.J., 2010. Application of bioassays in toxicological hazard, risk and impact assessments of dredged sediments. Mar. Pollut. Bull. 60, 2026–2042.

SEPA, 1999. Regulation and monitoring of marine cage fish farming in Scotland. In: SEPA (Ed.), A Procedures Manual Version 1.0. Scottish Environment Protection Agency, Stirling, pp. 6.

Shieh, J.N., Chao, M.R., Chen, C.Y., 2001. Statistical comparisons of the no-observed-effect concentration and the effective concentration at 10% inhibition (EC_{10}) in algal toxicity tests. Water Sci. Technol. 43, 141–146.

Stigebrandt, A., Aure, J., Ervik, A., Kupka, P., 2004. Regulating the local environmental impact of intensive marine fish farming. III. A model for estimation of the holding capacity in the modelling-ongrowing fish farm-monitoring system. Aquaculture 234, 239–261.

Tello, A., Corner, R.A., Telfer, T.C., 2010. How do land-based salmonid farms affect stream ecology? Environ. Pollut. 158, 1147–1158.

Tueros, I., Rodríguez, J.G., Borja, A., Solaun, O., Valencia, V., Millán, E., 2008. Dissolved metal background levels in marine waters, for the assessment of the physico-chemical status, within the European Water Framework Directive. Sci. Total Environ. 407, 40–52.

Underwood, A.J., 1997. Experiments in Ecology. Their Logical and Interpretation using Analysis of Variance, 8th ed. Press Syndicate of the University of Cambridge, Cambridge.

USEPA, 2000. Method guidance and recommendations for whole effluent toxicity (WET) testing. In: USEPA (Ed.), , United States Environmental Protection Agency, Washington, pp. 60.

Van der Grinten, E., Pikkemaat, M.G., Van den Brandhof, E.-J., Stroomberg, G.J., Kraak, M.H.S., 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphere 80, 1–6.

Viana, I., Carballeira, C., De Orte, M., Carballeira, A., . Assessing the toxicity of chemical compounds associated to land-based marine fish farms through the miniaturized bioluminiscence test with *V. fischeri*. Toxicology, submitted for publication.

Villares, R., Carballeira, A., 2004. Nutrient limitation in macroalgae (Ulva and Enteromorpha) from the Rías Baixas (NW Spain). Mar. Ecol. 25, 225–243.

Wang, F., Leung, A.O.W., Wu, S.C., Yang, M.S., Wong, M.H., 2009. Chemical and ecotoxicological analyses of sediments and elutriates of contaminated rivers due to e-waste recycling activities using a diverse battery of bioassays. Environ. Pollut. 157, 2082–2090.

Wessa, P., 2008. Kernel Density Estimation (v1.0.6) in Free Statistics Software (v1.1.23-r6), 1.1.23 Ed. Office for Research Development and Education.

Villares, R., Carballeira, A., 2006. Trophic categorization in the Rías Baixas (NW Spain): nutrients in water and in macroalgae. Sci. Mar. 70, 89–97.

Anexo IV

Marine Pollution Bulletin 62 (2011) 2633–2641

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Linking $\delta^{15}N$ and histopathological effects in molluscs exposed *in situ* to effluents from land-based marine fish farms

C. Carballeira [a,*], J. Espinosa [b], A. Carballeira [c]

[a] Grupo de Ecotoxicología Marina, Dpt° Química Física, Facultad de Ciencias del Mar (UCA), Spain
[b] Departamento de Fisiología, Facultad de Farmacia, Universidad de Santiago de Compostela, Spain
[c] Grupo de Ecotoxicología, Área de Ecología, Fac. Biología (USC), Spain

ARTICLE INFO

Keywords:
Mytilus galloprovincialis
Venerupis pullastra
Fucus vesiculosus
Codium tomentosum
Monitoring
Branchial exfoliation

ABSTRACT

Histopathological alterations can indicate time-integrated impacts on organisms stemming from alterations at lower biological organisation levels. Long-term (native mussels) and short-term (transplanted clams) changes in the tissues of molluscs exposed to the effluents from two land-based marine fish farms (LBMFFs) were determined. Histological alterations were related to the $\delta^{15}N$ isotopic signal measured in mussels and macroalgae.

Effluents from LBMFFs were found to cause severe and moderate gill filament exfoliation in clams and mussels, respectively. Some transplanted clams showed severe degrees of hemocytic phagocytosis in gonads and connective tissue. In an attempt to semi-quantitatively summarize the observed histopathological alterations, a weighted index of damage (WID) was calculated for each type of alteration, species and sampling site. The WID was clearly related to the $\delta^{15}N$ descriptor of exposure. Further studies aimed at standardizing this relationship may establish critical thresholds of the descriptor for its implementation within environmental monitoring plans for LBMFFs.

1. Introduction

Worldwide development of pisciculture has been possible due to improvements in feed, methods and structures, increased knowledge of farmed species and the increased international demand for fish (Read and Fernandes, 2003).

The use of anaesthetics, antibacterial, antiseptic and antiparasitic agents as well as anti-fouling, cleaning and disinfection products is necessary in aquaculture facilities, in order to maintain and guarantee a certain level of aquatic production (Burridge et al., 2010; Costello et al., 2001; Douet et al., 2009). The biocides and metabolic wastes from fish farm effluents may alter the species and communities in the receiving environment.

Bivalve molluscs, such as clams and mussels, may be affected by this type of waste. These filter feeders are widely distributed and abundant, they tend to bioaccumulate pollutants and are sedentary and easy to sample. These characteristics make them valuable sentinels in pollution monitoring programmes for studying the health of coastal and estuarine environments (e.g. The Mussel Watch Programme; Goldberg, 1986)).

The presence of contaminants can alter bivalve molluscs from biochemical to physiological level. Such alterations can be measured as exposure and effect biomarkers (Huggett, 1992; Ramos-Gómez et al., 2011). Sublethal responses are more sensitive than lethal measurements, and may enable identification of the real extent of environmental impacts (Riba et al., 2004).

Studies of histocytopathological alterations in target tissues of bivalves have been recommended as biomarkers in marine pollution biomonitoring. These alterations are sensitive to a wide range of contaminants (Au, 2004), and they indicate the status of target tissues, providing a general view of the damage received by molluscs. Analysis of histopathological alterations in the tissues of native mussels (Aarab et al., 2008; Wedderburn et al., 2000) and transplanted clams (Morales-Caselles et al., 2008; Nasci et al., 1999) are of increasing interest in marine pollution monitoring because of their sensitivity, availability and commercial relevance.

The histopathological analysis has shown to be a good method for *in situ* assessment of toxic effects in the short and long term (Handy et al., 2002; Morales-Caselles et al., 2008; Watermann et al., 2008).

The gills and the digestive gland of bivalves play an important role in food collection, absorption and digestion, and they are directly exposed to environmental contaminants. Gills filter water in order to obtain nutrients, and so are in continuous contact with water pollutants. The digestive gland of molluscs is the main

* Corresponding author. Tel.: +34 956016423; fax: +34 956016040.
E-mail addresses: carlos.carballeira@uca.es (C. Carballeira), joaquin.espinosa@usc.es (J. Espinosa), alejo.carballeira@usc.es (A. Carballeira).

0025-326X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2011.09.034

centre for metabolic regulation. It participates in the mechanisms of immune defence and homeostatic regulation of the internal medium, as well as in the processes of detoxification and elimination of xenobiotics (Moore and Icarus Allen, 2002). Exposure to contaminants may cause cell damage. In the case of gills, epithelial cells may disappear, while hemocytic phagocytosis may occur in both organs, as well as in the kidney, gonad and somatic tissues. The loss of epithelial cells and hemocytic phagocytosis may lead to tissue dysfunction and have detrimental effects on the individual health status.

Several authors have described the degree of exposure to organic pollution by analysing the stable isotopic ratio of nitrogen ($\delta^{15}N$) in organisms. Nitrogen has two stable isotopes, a light isotope, ^{14}N, and a heavier isotope, ^{15}N, which occur in a constant proportion in the atmosphere (99.635% and 0.365%, respectively) (Nier, 1950). Isotopic abundance is reported on a delta scale (δ), which indicates the deviation (expressed in ‰) of the isotopic composition of a sample from an internationally accepted standard: the air (Robinson, 2001). The anthropogenic input of N alters the background levels of $\delta^{15}N$ in marine systems (Heaton, 1986), and therefore $\delta^{15}N$ can be used to trace and quantify organic contamination from aquaculture activities (Dolenec et al., 2006; García-Sanz et al., 2010).

The inorganic nitrogen in the water is ingested (animals) or absorbed (primary producers), accumulated and immobilized in tissues of aquatic organisms in the vicinity of the farm. This enables integration of the $\delta^{15}N$ source signal over time (Costanzo et al., 2001; Lobban and Harrison, 1994; McClelland et al., 1997; Vosz and Struck, 1997). The ratio of $\delta^{15}N$ is temporally stable in natural conditions, and variations in the isotopic signal are mainly due to the influence of human activities (Savage and Elmgren, 2004). At present, the isotopic signal from the tissues of some species of molluscs and macroalga are well known (Costanzo et al., 2001; Deutsch and Voss, 2006; Mazzola and Sarà, 2001; Oevelen et al., 2009; Riera et al., 2000; Sará, 2006; Savage, 2005; Savage and Elmgren, 2004; Tewfik et al., 2005; Viana et al., 2011; Vizzini and Mazzola, 2004). For this reason, both native and transplanted molluscs and macroalgae have been used as biomonitors in different polluted environments affected by organic contamination from land based marine fish farms (LBMFFs). However, although the $\delta^{15}N$ in native organisms is a good descriptor of exposure to discharges from LBMFFs, the relations between descriptor values and effect on the target organisms must be established in order to estimate the real impact.

The objectives of this study were: (1) to determine histopathological alterations in tissues of two mollusc species after long-term exposure (native mussels) and short-term exposure (transplanted clams) to effluents from LBMFFs, in comparison with the selected reference histological parameters; (2) to evaluate the suitability of the histopathological measurements in both native mussels and transplanted clams as monitoring tools, according to the severity of the histological alterations and the maximum distance at which these alterations were observed; (3) to observe the relationship between histological alterations and the isotopic signal $\delta^{15}N$ in native organisms (mussels and macroalgae) for further use in monitoring the potential impact of effluents from LBMFFs.

2. Material and methods

2.1. Transfer and sampling

The study was performed in two areas of the Galician Coast (NW Spain) in July 2008. Two turbot (*Psetta maxima*) LBMFFs were selected. These farms are located at Lira, A Coruña (annual production of 1194 ty^{-1}), and Xove, Lugo (2250 ty^{-1}). There are no other close sources of organic pollution at either site, but the hydrodynamic characteristics are different. Five sampling sites (SS) were established at each fish farm site, along a non-linear gradient starting at the emission point and moving further away in the direction of the main current to a distance where farm effects were negligible (SS E or reference site) (Fig. 1).

The clam *Venerupis pullastra* Montagu, 1803 and the mussel *Mytillus galloprovincialis* Lamarck, 1819 were chosen for the study because of their ecological and commercial relevance in the Galician coast. Mussels are abundant on the characteristic rocky substrate of the study areas and easy to sample. Conversely, the habitat of clams is the sandy mud bottom of the rias, therefore, they are not present in the study sites. For this reason, transplantation methods were required.

Clams are considered useful within integrated multitrophic aquaculture systems, in fact, their suitability has already been studied with effluents of this particular type of aquaculture (Jara-Jara et al., 1997). However, organism suitability was assessed using growth, survival and biochemical composition, but biomarkers of effect were not considered. The use of clams in this work may provide new information about the adequacy of these organisms in multitrophic systems.

The use of two species and two experimental approaches allows encompassing a wider variety of interactions between biota and contaminants and provides a more precise assessment of the LBMFF impact.

Clams (35–40 mm shell length) were collected from a clean site located in the Ria de Muros-Noia and depurated for 7 days with clean natural sea water. A group of clams ($n = 30$) was used as reference. The other specimens were placed in cages ($n = 30$) and the cages were transferred to the different SS and kept there for 45 days.

Native mussels (75–80 mm shell length) were collected at all SS. Different species of macroalgae were also collected at Xove (*Fucus vesiculosus* L.) and Lira (*Codium tomentosum* Stackhouse, 1797). Sampling was carried out at low tide in the mesolittoral zone. Each site included 20 m of coastline where at least 30 mussels and 30 specimens of macroalgae (adhered to substrate) were collected systematically, following a zigzag line, with the aim of covering the degree of variability in the interindividual concentrations.

Once the exposure period for clams was completed, and mussels and macroalgae were harvested, the organisms were transported to the laboratory under controlled conditions (4 °C). Macroalgae were previously washed *in situ* with seawater and combined to make a composite sample at each SS. In the laboratory, clams and mussels assigned for histopathological determinations were immediately dissected. Macroalgae and mussels assigned for $\delta^{15}N$ analysis were processed for isotopic analysis.

2.2. Biomarkers of effect: assessment of histopathological alterations

The histopathological changes were studied in 10 randomly selected specimens of clams and mussels collected at each site. Gills were surgically removed from live specimens, and together with the rest of the soma, except the foot, were immediately fixed in Bouin's fixative for 1 h (gills) and 2 h (soma). Samples were then washed immediately with ethanol (70%) to remove all the fixative. The preparation process was carried out according to standard methods for bivalve molluscs (Howard et al., 2004). After dehydration in graded concentrations of ethanol, the samples were rinsed with xylene, impregnated and embedded in paraffin. Six histological sections of 3 μm were cut every 30 μm with a rotation microtome (Microm HM 360), and prepared for each specimen and region. Sections were stained with trichrome dye according to

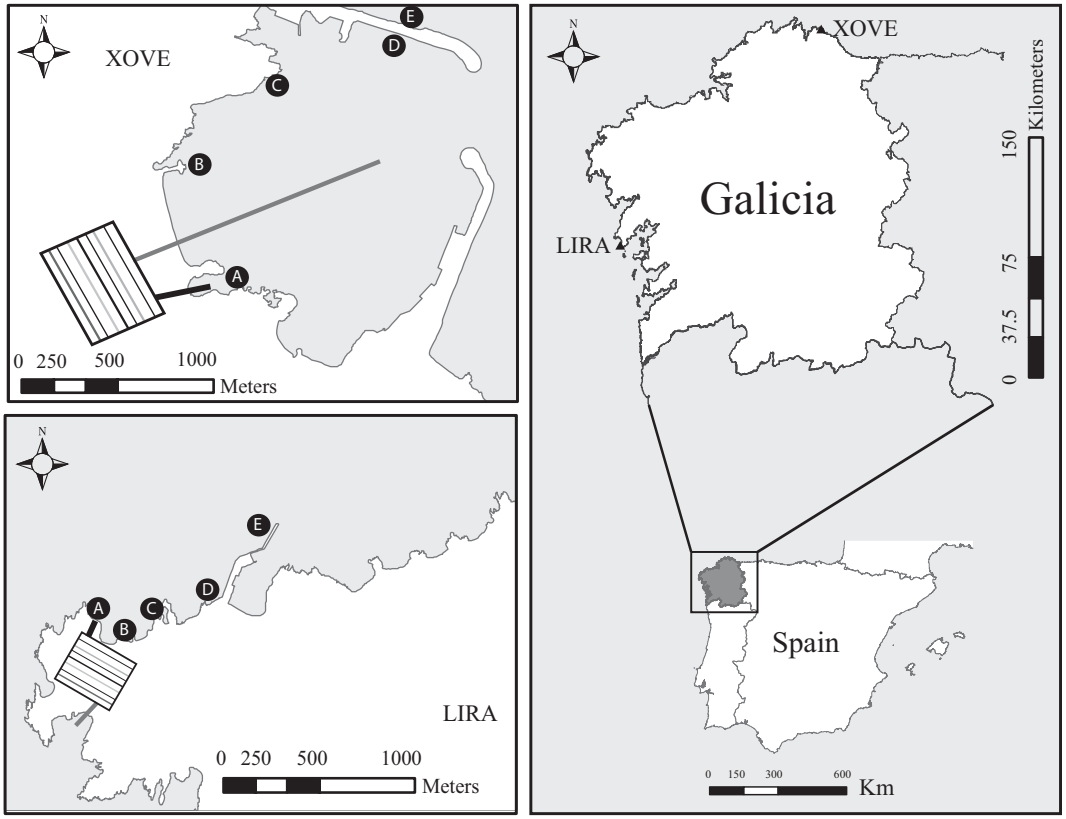


Fig. 1. Map of Spain showing the location of Galicia (NW Spain) and both fish farms, I: Lira, A Coruña and II: Xove, Lugo. Sampling sites (SS) are shown in the enlarged maps, and are designated from A to E according to the distance from the source of contamination. The arrows indicate the input (grey) and the output (black) of water in the land-based marine fish farms (hatched area).

Wheatley (1951), and were examined by light microscopy (Olympus BX-50 equipped with plan apochromatic objectives).

A set of measurements on the reference organisms and on those from the SS was performed to ensure consistency of results before ranking the histopathological alterations. Tissue damage in clams and mussels was quantified by counting the frequency of each alteration detected. The histopathological alterations were semi-quantitatively evaluated by ranking the severity of lesions in specimens as follows: 0 (none), 1 (slight), 2 (moderate) and 3 (severe) (Martín-Díaz et al., 2008; Riba et al., 2004). A general index of damage for each tissue (weighted index of damage, WID) was then calculated for each type of alteration, species and SS, with the aim of quantitatively summarizing the observed histological changes. The WID weights the degree of alteration by the frequency (%) observed in 10 individuals of a species from each SS, as follows: WID=[0 × % Not altered + 1 × % Slight Alteration + 2 × % Moderate Alt. + 3 × % Severe Alt.]/100. The WID for each type of histological damage of sites ranges from 0 (no damage was found in any individual) to 3 (when 100% of individuals had severe damage).

2.3. Stable isotope analysis ($\delta^{15}N$)

Ten randomly selected specimens of macroalgae and mussels were processed for isotopic analysis. Macroalgae were washed several times with abundant filtered seawater in order to remove, as efficiently as possible, any sediment and epiphytes. Old and damaged parts of the algae were discarded, and distal shoots (3 cm) were separated with a glass spatula to determine the isotopic concentrations. The mussel bodies were also separated from the shell and washed with filtered seawater to remove sediment. All samples of macroalgae and mussels were homogenized in a laboratory blender (Waring Blender 34BL99). The material was then dried at 45 °C in a forced air oven and homogenized once again in an ultra-centrifugal mill (Retsch ZM 100). Dry samples were stored at room temperature in glass vessels.

Aliquots (3 mg) of the dry samples of mussels and macroalgae were weighed out and packed in tin capsules. The capsules were stored in a desiccator until $\delta^{15}N$ analysis (carried out in the UTIA, University of A Coruña). The samples were combusted in an elemental analyser (FlashEA1112: ThermoFinnigan) coupled to an isotopic ratio mass spectrometer (Delta^{plus}: ThermoFinnigan). Acetanilide was used as the reference standard for quantifying the nitrogen content. Calibration of the reference gas for atmospheric ^{15}N was carried out with IAEA-N-1 ($(NH_4)_2SO_4$), IAEA-N-2 ($(NH_4)_2SO_4$) and IAEA-NO-3 (KNO_3) as standards. The isotopic ratios ($^{15}N/^{14}N$) in the samples were compared with the standard (atmospheric N_2), so that comparable proportions were obtained. The relative abundance of ^{15}N in the sample ($\delta^{15}N$) was calculated from the formula: $\delta^{15}N\ (‰) = [(R_{sample}/R_{standard}) - 1]*10^3$, where R is the $^{15}N/^{14}N$ ratio. The overall error was determined by use of analytical replicates. This constitutes a measure of precision as it is the coefficient between the standard deviation of the replicates and the number of replicates. The overall error of the replicates of 30 samples was 2%.

3. Results

3.1. Biomarkers of effect: histopathology

Transplanted clams at SS A (closest SS to the output channel) from Lira and Xove disappeared before sampling.

Two types of histopathological alterations were observed: branchial exfoliation (Fig. 2) and hemocytic phagocytosis (Fig. 3). The

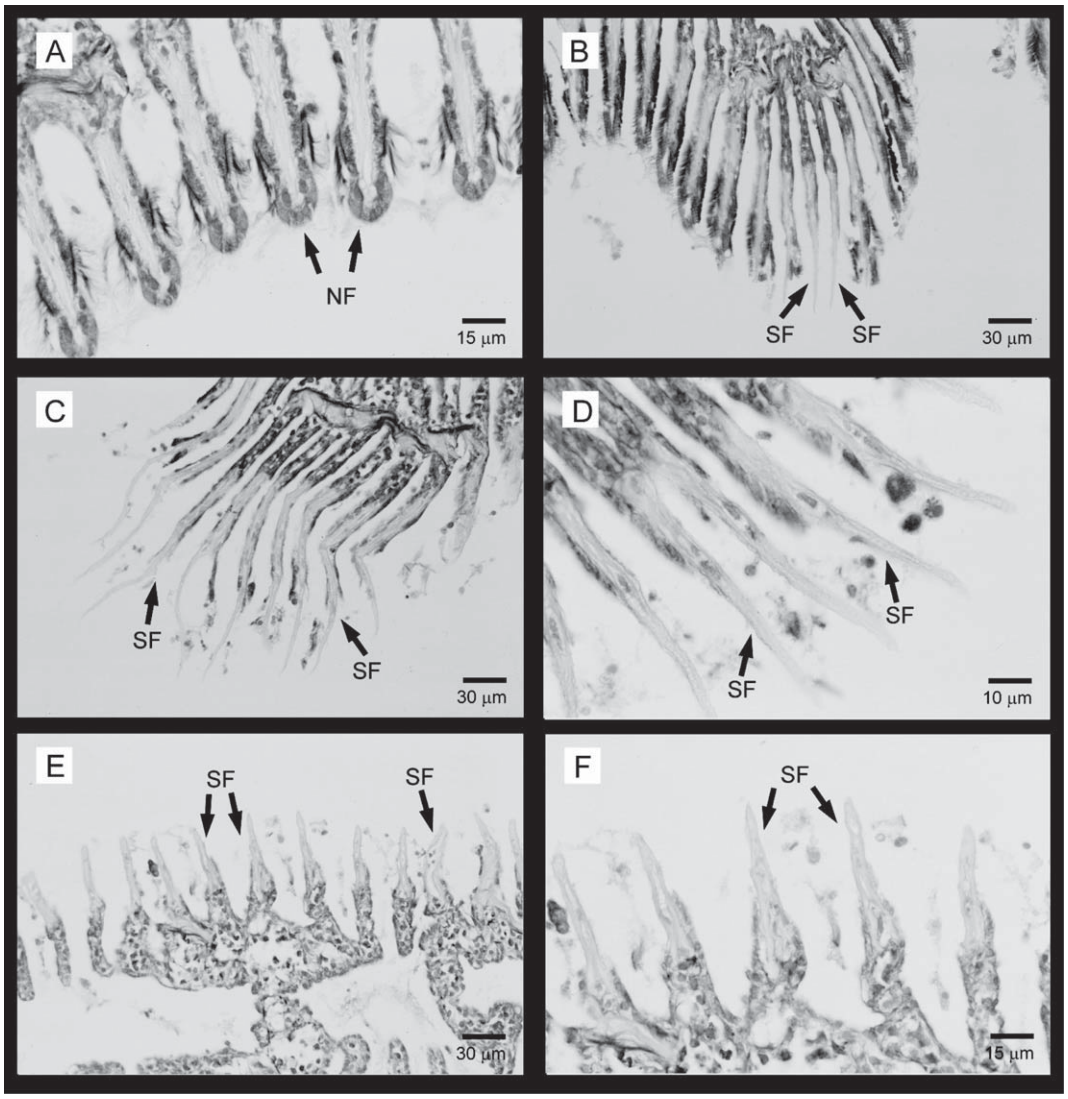


Fig. 2. Histopathological alterations in gills of clams and mussels. (A) Normal clam: the gill filaments have the normal density of ciliated epithelial cells; (B) slight alteration in clam: some of the branchial filaments are bare because of desquamation of ciliated epithelial cells and caliciform cells. The distal region of the filaments shows only the skeleton of collagen fibres, at the site of exfoliated epithelium; (C) severe alteration in clam: all branchial filaments are bare due to epithelial exfoliation; (D) magnification of severe histopathological alteration in clam: skeleton of collagen fibres without branchial epithelium; (E) severe alteration in mussel: branchial filaments are bare; (F) magnification of severe alteration in mussel: skeleton of collagen fibres without branchial epithelium. [NF: normal filament; SF: stripped filament].

frequency of individuals affected by different degrees of disturbances are shown in Table 1.

The histopathological results showed no alterations in the mussels located at distant SS (E), and in clams before being transplanted or after exposure at SS E. None of the alterations previously mentioned were found in the digestive system or the kidney.

Slight, moderate and intensely stripped gills were observed in clams (Fig. 2A–D). Clams showing branchial exfoliation (Fig. 2B) were found as far as 400 m away (SS D) from the output, at both farms, whereas other organs and tissues did not show any alteration. At Xove, transplanted clams showed severe branchial exfoliation (Fig. 2C and D) at SS B, moderate at SS C and slight at SS D (Fig. 2B).

Mussels rarely showed intense branchial exfoliation (Fig. 2E and F). Moderate and slight branchial exfoliation was observed in native mussels from Lira collected from SS A and B, respectively. Slight branchial exfoliation of native mussels was found at Xove (SS A, B and C).

Hemocytic phagocytosis (intense formation of clusters of phagocytic hemocytes) was found in gonadal follicles (Fig. 3A), gonoducts (Fig. 3B and C) and somatic tissue (Fig. 3D–F) from transplanted clams at Xove. Hemocytic phagocytosis in these clams even appeared at SS D and was severe at SS B.

In general, molluscs at the most exposed SS displayed intense exfoliation of ciliated epithelial cells and caliciform cells from the gill filaments.

3.2. Stable isotope analysis ($\delta^{15}N$)

The values of $\delta^{15}N$ in mussel and macroalgae collected at the different SS in the surroundings of the LBMFFs are shown in Table 2. The SS are ordered from A to E, according to increasing distance from the output (Fig. 4). Enrichment of $\delta^{15}N$ in organisms was observed at both fish farms, with decreasing distance from the emission points. Higher values of $\delta^{15}N$ were observed at Xove, probably because of a higher load of effluents combined with a lower rate of water renewal.

3.3. Relating ^{15}N in macroalgae and biomarkers of effect in molluscs

The weighted index of histological damage (WID) observed in clams and mussels from each farm varied with the isotopic signal

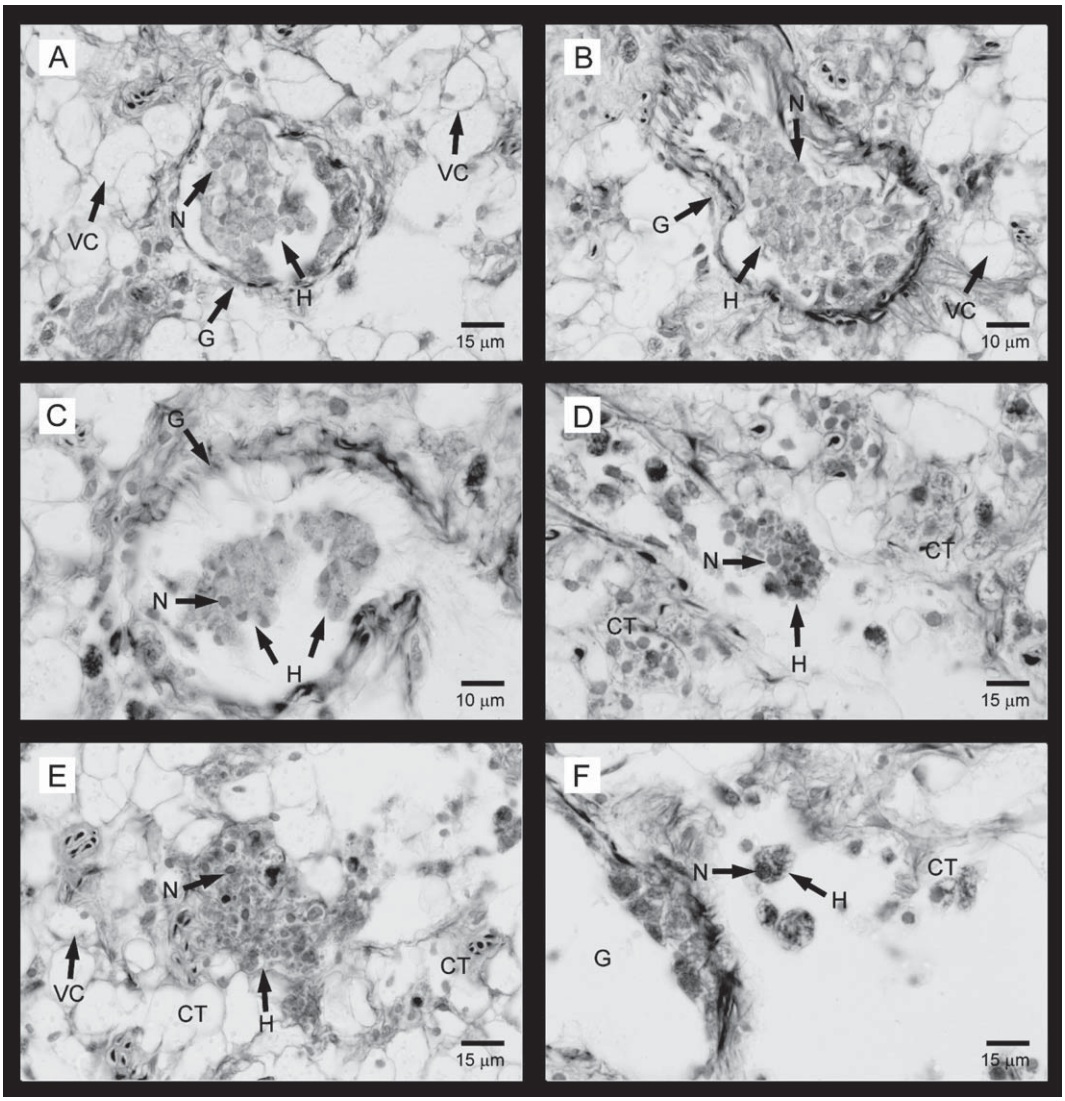


Fig. 3. Hemocytic phagocytosis in clam. (A) Hemocytic phagocytosis in gonadal follicle (without sexual cells) with an abnormal large cluster of hemocytes within the follicle. The cytoplasm of hemocytes is densely filled with yellow phagocytized material; (B) hemocytic phagocytosis inside a gonoduct with clusters of hemocytes and yellow phagocytized material; (C) hemocytic phagocytosis inside another gonoduct with two clusters; (D) hemocytic phagocytosis in somatic connective tissue; (E) cluster from connective somatic tissue with a high density of hemocytes and phagocytized material; (F) free hemocytes, with no phagocytic material, in the somatic connective tissue. [CT: somatic connective tissue; G: gonad follicle; H: hemocytes; N: hemocyte nucleus; VC: vesicular cells of the connective somatic tissue].

($\delta^{15}N$) determined in mussels and macroalgae (Fig. 4). A pollution gradient was observed at both LBMFFs, but the changes in histopathological effects were different for each biomarker and scenario. Moderate and severe branchial exfoliation of clams and mussels started at lower values of $\delta^{15}N$ at Lira than at Xove, but changes in this alteration were later more pronounced at Xove (Fig. 4). Histopathological alterations were correlated with lower $\delta^{15}N$ values in mussel than in macroalgae (Fig. 4). Only clams from Xove were affected by hemocytic phagocytosis and this alteration followed the same trend as branchial exfoliation in clams.

4. Discussion

4.1. Biomarkers of effects: histopathology

Most of the effects observed in this study can also be found in different species of marine molluscs exposed to different inorganic and organic contaminants, parasitic or infectious diseases, nutritional stress or physicochemical disorders (Aarab et al., 2008; Morales-Caselles et al., 2008; Rodríguez de la Rua et al., 2005).

Epithelial exfoliation and hemocytic phagocytosis may greatly compromise reproduction and survival of molluscs in the short and mid-term.

The severe epithelial exfoliation of the gill filaments of some individuals will lead, in the short term, to difficulties in filtering food, and in the mid term, to breathing problems, seriously compromising survival of the animals. Likewise, the lack of mucus needed for filtration and ingestion of food particles is provoked by loss of caliciform cells. On the other hand, the high maintained energy costs, derived from hemocytic phagocytosis, together with the formation of clusters will provoke, in the mid-long term, a decline in gonadal development and animal growth (Barber and Blake, 1981; Delaporte et al., 2006; Griffiths and King, 1979).

There are two sources of phagocytosis: (1) exogenous phagocytosis, when animals are exposed to particulate pollution and (2) endogenous phagocytosis from altered tissue material. These processes are irreversible. The hemocytes involved in phagocytosis do not recover, so the animal must continue producing them to maintain the hemocyte defence barrier (Donaghy et al., 2009). This defensive structure is developed as a consequence of other hard

Table 1
Branchial exfoliation and phagocytic haemocytosis in native mussels (*M. galloprovincialis*) and transplanted clams (*V. pullastra*) at different sampling sites (from A to E), located in the vicinity of the Lira and Xove LBMFFs.

Histological alteration		Native mussel (*M. galloprovincialis*)				Transplanted clam (*V. pullastra*)					
		Branchial exfoliation				Branchial exfoliation				Phagocytic haemocytosis	
LBMFF		LIRA		XOVE		LIRA		XOVE		XOVE	
SS	Degree of Alteration[a]	Frequency (%)	WID[b]	Frequency (%)	WID[b]	Frequency (%)	WID[b]	Frequency (%)	WID[b]	Frequency (%)	WID[b]
A	0	30	1.1	30	0.9	–	–	–	–	–	–
	1	40		50		–		–		–	
	2	30		20		–		–		–	
	3	0		0		–		–		–	
B	0	60	0.8	40	0.8	0	2.4	0	2.1	10	2.4
	1	40		40		10		0		10	
	2	0		20		40		30		40	
	3	0		0		50		70		40	
C	0	100	0	70	0.3	10	2.2	50	0.8	40	0.8
	1	0		30		10		20		20	
	2	0		0		30		30		40	
	3	0		0		50		0		0	
D	0	100	0	100	0	30	1.2	80	0.2	70	0.2
	1	0		0		30		20		30	
	2	0		0		30		0		0	
	3	0		0		10		0		0	
E	0	100	0	100	0	100	0	100	0	100	0
	1	0		0		0		0		0	
	2	0		0		0		0		0	
	3	0		0		0		0		0	

[a] Degree of histological alteration: 0 none, 1 slight, 2 moderate, 3 severe.
[b] WID: Weighted Index of Damage.

Table 2
Values of the $\delta^{15}N$ isotopic ratio (‰) in macroalgae (*C. tomentosum* and *F. vesiculosus)* and mussels (*M. galloprovincialis)* collected at sampling sites (SS) A, B, C, D and E, in the surroundings of the Lira and Xove LBMFFs.

LBMFF[b]	LIRA		XOVE	
SS[a]	*C. tomentosum*	*M. galloprovincialis*	*F. vesiculosus*	*M. galloprovincialis*
A	9.93 ± 0.19	9.14 ± 0.17	10.67 ± 0.20	10.18 ± 0.11
B	9.84 ± 0.17	9.22 ± 0.18	11.17 ± 0.21	11.48 ± 0.23
C	9.30 ± 0.18	8.60 ± 0.15	9.77 ± 0.17	9.11 ± 0.16
D	8.14 ± 0.16	7.60 ± 0.21	9.57 ± 0.18	8.89 ± 0.14
E	5.57 ± 0.11	6.87 ± 0.16	5.61 ± 0.12	5.77 ± 0.13

[a]SS: Sampling sites.
[b]LBMFF: Land-based marine fish farm.

tissue changes that are indicated by molecular biomarkers (Aarab et al., 2008; Jenny et al., 2002; Morales-Caselles et al., 2008).

Transplanted clams exhibited more severe lesions than native mussels, and these injuries were observed at a greater distance from the farm output than those recorded in native mussels. Therefore, this methodology appeared to be more sensitive than the use of native mussels when assessing the impact of LBMFFs.

Significant physiological differences have been found amongst bivalve species, which may determine their particular sensitivity to the effects of environmental contaminants. Differences in the filtration rates, the diet (Riisgaard, 1988), the particle retention efficiency (Winter, 1978), the capacity to accumulate xenobiotics (Baudrimont et al., 2005; De Luca-Abbott et al., 2005; Im et al., 2004), the contaminant detoxification, the storing estrategies (Pellerin and Amiard, 2009) and the ability to repair damages, leads to a distinct vulnerability and different levels of exposure to dissolved or adsorbed contaminants. Besides, mussels filtered according to the tidal dynamics, while transplanted clams are submerged the whole day. This may have intensified the clam exposure to the potentially contaminated water. Native mussels had been chronically exposed to water affected by the fish farm discharges, therefore, they may have developed adaptation mechanisms against pollution. Transplantation approaches have been found to be more sensitive than native biota studies because they discard the phenomena of adaptation, which are translated into sublethal chronic pollution levels (Da Ros and Nesto, 2005).

4.2. Linking ^{15}N and biomarkers of effect in molluscs

In situ measures of effects represent an important line of evidence in ecological risk assessment (Baird et al., 2007; Crane et al., 2007). The application of toxicity bioassays under field conditions has been proposed by many authors in order to assess responses in aquatic populations under realistic environmental conditions (Martín-Díaz, 2004; McCarthy and Shugart, 1990). However, field experiments are expensive and the results are difficult to interpret because of the complexity and fluctuations in environmental interactions, which may alter the toxicity (Crane et al., 2007). Both farms breed and fatten turbot with the same feed, and should therefore discharge similar metabolic wastes. However, other products may differ, for example those used for disease prevention and disinfection. Rey-Asensio et al. (2010) studied pollution from four LBMFFs (including Lira) by biomonitoring the area of influence with native organisms (*F. vesiculosus, C. tomentosum, M. galloprovincialis, Anemone sulcata*) and *Saccharine saccharine* transplants. Biomonitoring was considered necessary

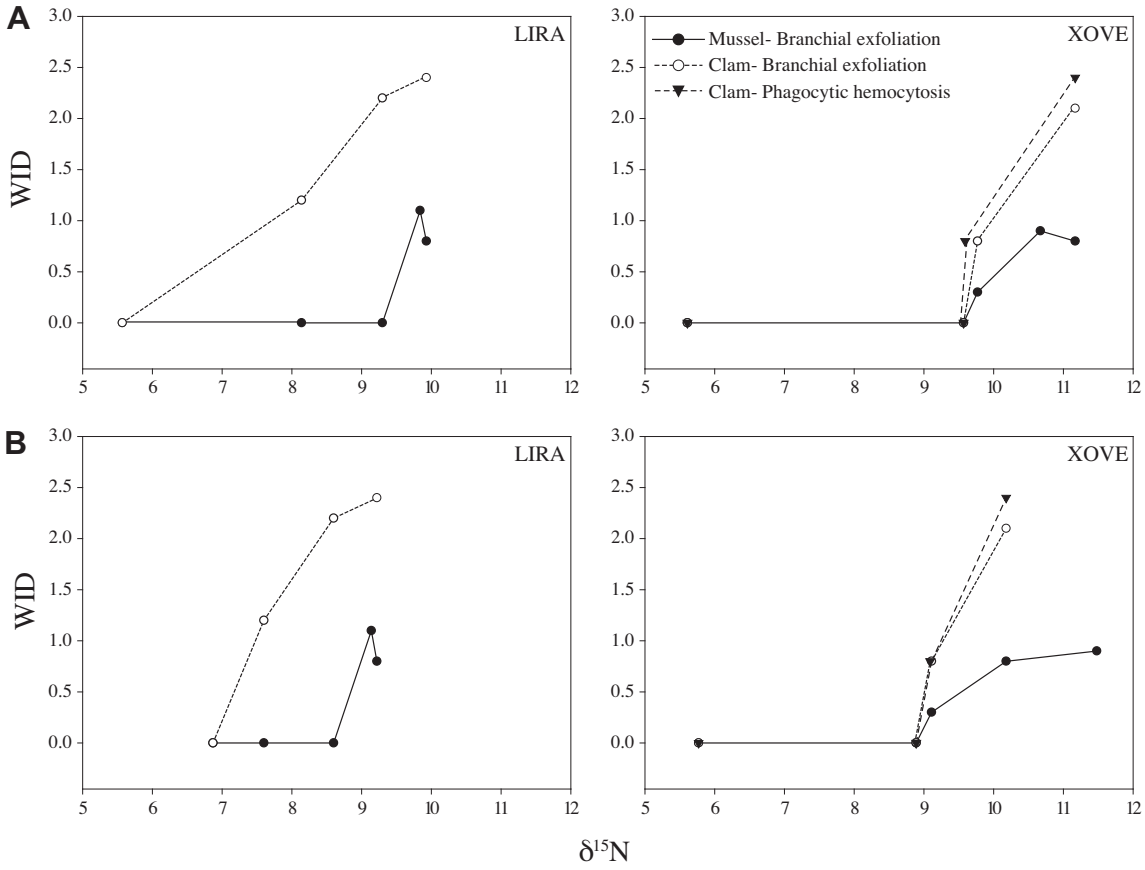


Fig. 4. Variation in Weighted Index of Histological Damage (WID) (including branchial exfoliation and hemocytic phagocytosis) from molluscs in relation to the isotopic signal from (A) macroalgae and (B) mussels affected by effluents from the Lira and Xove LBMFFs.

due to the high temporal variability and the degree of dilution of pollutants from the effluents. Bioaccumulation of metals, antibiotics and pesticides clearly varied according to the LBMFF and the type of biomonitor selected. This could explain the differences on the WID-δ^{15}N relations between scenarios (Fig. 5).

The values of the isotopic signal from macroalgae collected at the reference SS (the farthest from the emission source) are within the regional background range (5.48 ± 1.18‰) (Viana et al., 2011). This highlights the influence of discharges from farms on the marine environment.

Viana et al. (2010) reported no significant differences in the δ^{15}N in three macroalgae of the genus *Fucus* sp. and *C. tomentosum* from the Galicia coast (NW Spain), grown with the same nutrients. This indicates that the different species of macroalgae can be used indiscriminately, which may facilitate the search for local biomonitors and promote their use (Carballeira and Carballeira, 2009). There was a clear isotopic signal in macroalgae affected by discharges from LBMFFs, so δ^{15}N appears to be a good descriptor of the degree of exposure and area of influence. δ^{15}N analysis should be included in environmental monitoring plans for fish farms because it provides information on the main environmental impact from this activity, i.e. potential eutrophication.

The δ^{15}N values in macroalgae and mussels were similar and followed the same trend at both LBMMFs. The δ^{15}N in algae and mussels from the area of influence of eight Galician LBMFFs (two included in this study) were closely correlated [$r^2 = 0.79$; Sig = 0024; $n = 19$ (unpublished data)]. In general, the signal was stronger in macroalgae than in mussels, although when the pollutant load decreased, the effect appeared to be reversed, as previously reported by Riera et al. (2000). Ranges of variation of δ^{15}N in macroalgae were also higher than in mussels. Since the levels and variability of δ^{15}N in macroalgae were found to be higher, these organisms appeared to be better at discriminating the degree of exposure to pollution.

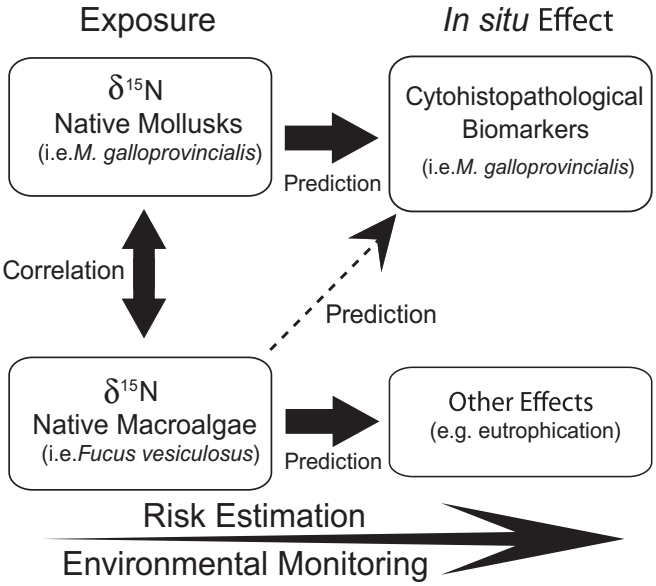


Fig. 5. Relation between histopathological alterations in molluscs and the δ^{15}N isotopic signal in mussels and macroalgae. The δ^{15}N isotopic signal from macroalgae is a good descriptor of exposure to waste water effluents, and therefore facilitates environmental surveillance of land-based marine fish farms.

When analyzing the apex of macroalgae, the exposure time is normalized for all individuals, while the $\delta^{15}N$ signal in molluscs largely depends on the condition index, which is difficult to standardize in native organisms. Another advantage of macroalgae is the possibility of carrying out retrospective studies of contamination from fish farms by analysis of tissue (apexes) of different ages (i.e. exposed at different times) (Savage and Elmgren, 2004).

5. Conclusions

The study of histopathological alterations in native and transplanted molluscs affected by effluents from LBMFFs (in NW Spain) is a useful biotool for monitoring the bioavailability and effects of contaminants, because of the realistic conditions of the exposure, the sensitivity and the robustness of the sublethal responses.

Analysis of $\delta^{15}N$ in native organisms has been found to be a good descriptor of exposure. For the first time, histopathological biomarkers (biomarkers of effect), measured in molluscs, have been associated with an exposure descriptor ($\delta^{15}N$), determined for macroalgae and molluscs. This may enable evaluation of potential effects at tissue level (which is usually complex and time consuming), by conducting a simple and rapid $\delta^{15}N$ analysis. Stable isotope analysis should therefore be included in order to assess the potential impact and the ecological integrity, within environmental monitoring plans for LBMFFs. However, further study about the relationship between the $\delta^{15}N$ signal and the histopathological biomarkers in transplanted organisms, in addition to those determined in native biota, is encouraged to provide a more complete and reliable assessment of the usefulness of $\delta^{15}N$ as a biomonitoring tool.

In order to validate the descriptor-effect relationship, for practical application, the study should be extended to other scenarios in order to establish a critical value of the descriptor indicating when the damage is irreversible or survival of molluscs is significantly threatened. In the case of the Galician coast, *M. galloprovincialis* should be selected as the native mollusc because of its abundance on the coasts where LBMFFs are located.

This study was not undertaken to elucidate the causal factors of histopathological alterations, which may be the consequence of a variety of pollutants. The aim of the study was to simplify technical aspects and improve the cost-effectiveness in the design of an appropriate environmental monitoring plan for this type of fish farm.

Acknowledgements

The present study was partly financed by the Spanish Government's National Plan for Marine Culture (JACUMAR, 2008): "Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)". Carlos Carballeira is grateful for funding from the University of Cadiz Predoctoral Fellowship Programme (Spain).

References

Aarab, N., Pampanin, D.M., Naevdal, A., Oysaed, K.B., Gastaldi, L., Bechmann, R.K., 2008. Histopathology alterations and histochemistry measurements in mussel, *Mytilus edulis* collected offshore from an aluminium smelter industry (Norway). Mar. Pollut. Bull. 57, 569–574.

Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar. Pollut. Bull. 48, 817–834.

Baird, D.J., Brown, S.S., Lagadic, L., Liess, M., Maltby, L., Moreira-Santos, M., Schulz, R., Scott, G.I., 2007. *In situ*-based effects measures: Determining the ecological relevance of measured responses. Integr. Environ. Assess. Manage. 3, 259–267.

Barber, B.J., Blake, N.J., 1981. Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (say). J. Exp. Mar. Biol. Ecol. 52, 121–134.

Baudrimont, M., Schäfer, J., Marie, V., Maury-Brachet, R., Bossy, C., Boudou, A., Blanc, G., 2005. Geochemical survey and metal bioaccumulation of three bivalve species (*Crassostrea gigas, Cerastoderma edule* and *Ruditapes philippinarum*) in the Nord Médoc salt marshes (Gironde estuary, France). Sci. Total Environ. 337, 265–280.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K., 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306, 7–23.

Carballeira, C., Carballeira, A., 2009. Consideraciones para un plan integral de vigilancia ambiental de las piscifactorías marinas instaladas en tierra, in: M. Rey Méndez, J.F.C., M. Izquierdo Rodriguez y A. Guerra Díaz (Ed.), XII Foro dos Recursos Mariños e da Acuicultura das Rías Galegas. USC, O Grove, pp. 101–126.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., Loneragan, N.R., Thomas, M., 2001. A new approach for detecting and mapping sewage impacts. Mar. Pollut. Bull. 42, 149–156.

Costello, M.J., Grant, A., Davies, I.M., Cecchini, S., Papoutsoglou, S., Quigley, D., Saroglia, M., 2001. The control of chemicals used in aquaculture in Europe. J. Appl. Ichthyol. 17, 173–180.

Crane, M., Burton, G.A., Culp, J.M., Greenberg, M.S., Munkittrick, K.R., Ribeiro, R., Salazar, M.H., St-Jean, S.D., 2007. Review of aquatic *in situ* approaches for stressor and effect diagnosis. Integr. Environ. Assess. Manage. 3, 234–245.

Da Ros, L., Nesto, N., 2005. Cellular alterations in *Mytilus galloprovincialis* (LMK) and *Tapes philippinarum* (Adams and Reeve, 1850) as biomarkers of environmental stress: Field studies in the Lagoon of Venice (Italy). Environ Inter. 31, 1078–1088.

Delaporte, M., Soudant, P., Lambert, C., Moal, J., Pouvreau, S., Samain, J.-F., 2006. Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle. Aquaculture 254, 571–582.

De Luca-Abbott, S.B., Richardson, B.J., McClellan, K.E., Zheng, G.J., Martin, M., Lam, P.K.S., 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters. Mar. Poll Bull. 51, 694–707.

Deutsch, B., Voss, M., 2006. Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: Results from *in-situ* incubation experiments. Sci. Total Environ. 366, 799–808.

Dolenec, T., Lojen, S., Lambasa, S., Dolenec, M., 2006. Effects of fish farm loading on sea grass *Posidonia oceanica* at Vrgada Island (Central Adriatic): a nitrogen stable isotope study. Isotopes Environ. Health Stud. 42, 77–85.

Donaghy, L., Lambert, C., Choi, K.-S., Soudant, P., 2009. Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current knowledge and future prospects. Aquaculture 297, 10–24.

Douet, D.G., Le Bris, H., Giraud, E., 2009. Environmental aspects of drug and chemical use in aquaculture: An overview. Options Méditerranéennes 86, 105–126.

García-Sanz, T., Ruiz-Fernández, J.M., Ruiz, M., García, R., González, M.N., Pérez, M., 2010. An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. Mar. Environ. Res. 70, 189–200.

Goldberg, E.D., 1986. The Mussel Watch concept. Environ. Monit. Assess. 7, 91–103.

Griffiths, C.L., King, J.A., 1979. Energy expended on growth and gonad output in the ribbed mussel *Aulacomya ater*. Mar. Biol. 53, 217–222.

Handy, R.D., Runnalls, T., Russell, P.M., 2002. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in southern england that meet the freshwater fisheries directive. Ecotoxicology 11, 467–479.

Heaton, T.H.E., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: A review. Isot. Geosci. 59, 87–102.

Howard, D.W.L., E.J.; Keller, B.J.; Smith, C.S., 2004. Histological techniques for marine bivalve mollusks and crustaceans, 2 ed. National Oceanic and Atmospheric Administration, Silver Spring.

Huggett, R.J., 1992. Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publishers, Boca Raton.

Im, S.H., Strause, K.D., Giesy, J.P., Chang, Y.S., Matsuda, M., Wakimoto, T., 2004. Concentrations and accumulation profiles of polychlorinated dibenzo-p-dioxins and dibenzofurans in aquatic tissues, and ambient air from South Korea. Chemosphere 55, 1293–1302.

Jara-Jara, R., Pazos, A.J., Abad, M., Garcia-MartinSanchez, I.O., Sanchez, J.I., 1997. Growth of clam seed (*Ruditapes decussatus*) reared in the waste water effluent from a fish farm in Galicia (N W. Spain). Aquaculture 158, 247–262.

Jenny, M.J., Ringwood, A.H., Lacy, E.R., Lewitus, A.J., Kempton, J.W., Gross, P.S., Warr, G.W., Chapman, R.W., 2002. Potential indicators of stress response identified by expressed sequence tag analysis of hemocytes and embryos from the american oyster, *Crassostrea virginica*. Mar. Biotechnol. 4, 81–93.

Lobban, C.S., Harrison, P.J., 1994. Seaweed Ecology and Physiology. Press Syndicate of the University of Cambridge, Cambridge.

Martín-Díaz, M.L., 2004. Determinación de la calidad ambiental de sistemas litorales y de estuario de la Península ibérica utilizando ensayos de campo y laboratorio Chemistry-physics. Universidad de Cadiz, Cadiz, p. 330.

Martín-Díaz, M.L., Jiménez-Tenorio, N., Sales, D., DelValls, T.A., 2008. Accumulation and histopathological damage in the clam *Ruditapes philippinarum* and the crab *Carcinus maenas* to assess sediment toxicity in Spanish ports. Chemosphere 71, 1916–1927.

Mazzola, A., Sarà, G., 2001. The effect of fish farming organic waste on food availability for bivalve molluscs (Gaeta Gulf, Central Tyrrhenian, MED): stable carbon isotopic analysis. Aquaculture 192, 361–379.

McCarthy, J.F., Shugart, L.R., 1990. Biomarkers of environmental contamination. Lewis Publishers, California.

McClelland, J.W., Valiela, I., Michener, R.H., 1997. Nitrogen-stable isotope signatures in estuarine food webs: A record of increasing urbanization in coastal watersheds. Limnol. Oceanogr. 42, 930–937.
Moore, M.N., Icarus Allen, J., 2002. A computational model of the digestive gland epithelial cell of marine mussels and its simulated responses to oil-derived aromatic hydrocarbons. Mar. Environ. Res. 54, 579–584.
Morales-Caselles, C., Martín-Díaz, M.L., Riba, I., Sarasquete, C., DelValls, T.Á., 2008. Sublethal responses in caged organisms exposed to sediments affected by oil spills. Chemosphere 72, 819–825.
Nasci, C., Da Ros, L., Campesan, G., Van Vleet, E.S., Salizzato, M., Sperni, L., Pavoni, B., 1999. Clam transplantation and stress-related biomarkers as useful tools for assessing water quality in coastal environments. Mar. Pollut. Bull. 39, 255–260.
Nier, A.O., 1950. A Redetermination of the relative abundances of the isotopes of neon, krypton, rubidium, xenon, and mercury. Phys. Rev. 79, 450.
Oevelen, v.D.J., Soetaert, K.E.R., Franco, M.A., Moodley, L., IJzerloo, v.L.P., Vincx, M., Vanaverbeke, J., 2009. Organic matter input and processing in two contrasting North Sea sediments: insights from stable isotope and biomass data. Mar. Ecol. Prog. Ser. 380, 19–32.
Pellerin, J., Amiard, J.-C., 2009. Comparison of bioaccumulation of metals and induction of metallothioneins in two marine bivalves (*Mytilus edulis* and *Mya arenaria*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 150, 186–195.
Ramos-Gómez, J., Coz, A., Viguri, J.R., Luque, A., Martín-Díaz, M.L., Delvalls, T.A., 2011. Biomarker responsiveness in different tissues of caged *Ruditapes philippinarum* and its use within an integrated sediment quality assessment. Environ. Pollut. 159, 1914–1922.
Read, P., Fernandes, T., 2003. Management of environmental impacts of marine aquaculture in Europe. Aquaculture 226, 139–163.
Rey-Asensio, A., Carballeira, C., Viana, I.G., Carballeira, A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioacumulación de microcontaminantes, in: Rey-Méndez M., L.C., Fernández Casal J., Guerra A. (Ed.), Foro dos Recursos mariños e da Acuicultura das Rías galegas XIII. USC, O Grove, pp. 201-218.
Riba, I., González de Canales, M., Forja, J.M., DelValls, T.A., 2004. Sediment quality in the Guadalquivir estuary: sublethal effects associated with the Aznalcóllar mining spill. Mar. Pollut. Bull. 48, 153–163.
Riera, P., Stal, L.J., Nieuwenhuize, J., 2000. Heavy δ^{15}N in Intertidal Benthic Algae and Invertebrates in the Scheldt Estuary (The Netherlands): Effect of River Nitrogen Inputs. Estuar Coast Shelf Sci. 51, 365–372.
Riisgaard, H., 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Mar. Ecol. Prog. Ser. 45, 217–223.
Robinson, D., 2001. Δ^{15}N as an integrator of the nitrogen cycle. Trends Ecol. Evol. 16, 153–162.
Rodríguez de la Rua, A., Arellano, J.M., González de Canales, M.L., Blasco, J., Sarasquete, C., 2005. Accumulation of copper and histopathological alterations in the oyster *Crassostrea angulata*. Cienc. Mar. 31, 455–466.
Sará, G., 2006. Hydrodynamic effects on the origin and quality of organic matter for bivalves: an integrated isotopic, biochemical and transplant study. Mar. Ecol. Prog. Ser. 328, 65–73.
Savage, C., 2005. Tracing the influence of sewage nitrogen in a coastal ecosystem using stable nitrogen isotopes. AMBIO 34, 145–150.
Savage, C., Elmgren, R., 2004. Macroalgal (*Fucus vesiculosus*) δ^{15}N values trace decrease in sewage influence. Ecol. Appl. 14, 517–526.
Tewfik, A., Rasmussen, J.B., McCann, K.S., 2005. Anthropogenic enrichment alters A marine benthic food web. Ecology 86, 2726–2736.
Viana, I.G., Carballeira, C., Rey-Asensio, A., Carballeira, A., 2010. Evaluation of the interespecific differences in δ^{15}N in coexisting marine macroalgae, The 7th International Conference on Applications of Stable Isotope Techniques to Ecological Studies, Fairbanks (Alaska).
Viana, I.G., Fernández, J.A., Aboal, J.R., Carballeira, A., 2011. Measurement of δ^{15}N in macroalgae stored in an environmental specimen bank for regional scale monitoring of eutrophication in coastal areas. Ecol. Indic. 11, 888–895.
Vizzini, S., Mazzola, A., 2004. Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Mar. Pollut. Bull. 49, 61–70.
Vosz, M., Struck, U., 1997. Stable nitrogen and carbon isotopes as indicator of eutrophication of the Oder river (Baltic sea). Mar. Chem. 59, 35–49.
Watermann, B., Thomsen, A., Kolodzey, H., Daehne, B., Meemken, M., Pijanowska, U., Liebezeit, G., 2008. Histopathological lesions of molluscs in the harbour of Norderney, Lower Saxony, North Sea (Germany). Helgol. Mar. Res. 62, 167–175.
Wedderburn, J., McFadzen, I., Sanger, R.C., Beesley, A., Heath, C., Hornsby, M., Lowe, D., 2000. The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. Mar. Pollut. Bull. 40, 257–267.
Wheatley, W., 1951. A rapid staining procedure for intestinal amoebae and flagellates. Am. J. Clin. Pathol. 21, 990–991.
Winter, J.E., 1978. A review on the knowledge of suspension-feeding in lamellibranchiate bivalves, with special reference to artificial aquaculture systems. Aquaculture 13, 1–33.

Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos de fitoplancton

Carballeira[1] C., Rey-Asensio[2], A., Carballeira[2], A.

[1]Departamento de Química Física, Cátedra UNESCO/UNITWIN/WICOP, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain. Email: carlos.carballeira@uca.es.
[2]Grupo de Ecotoxicología, Área de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

Resumen

Existen escasos estudios de la dispersión y el impacto ecológico que tienen los vertidos de las piscifactorías marinas instaladas en tierra sobre la comunidad fitoplanctónica nativa confinada en bolsas de diálisis. Este impacto se caracteriza a través de las variaciones en las concentraciones e índices pigmentarios y en la fluorescencia clorofílica después del período de exposición de los trasplantes. Por otro lado, la señal isotópica $\delta^{15}N$ observada en la comunidad de fitoplancton después del período de exposición indica la dispersión gradual con la distancia de la carga de nutrientes emitida. Sin embargo, las respuestas biológicas observadas no concuerdan con los procesos de eutrofización observados en la bibliografía con la misma técnica pero en otros escenarios. Existen diversos factores que pueden estar alterando las respuestas; el efecto trasplante, la duración del período de exposición o un efecto combinado tóxico-trófico.

Palabras clave: *Aquaculture; Phytoplankton bioasssay, Nitrogen stable isotopes ($\delta^{15}N$); pigmentary indices; chlorophyll fluorescence*.

1. Introducción

La eutrofización es el proceso de enriquecimiento del agua con nutrientes que causa una aceleración del crecimiento de las algas y otras formas de plantas que produce una perturbación indeseable de la calidad del agua y del equilibrio de los organismos presentes en el agua (OSPAR, 2003). Excretas, heces y exceso de alimento enriquecen de nutrientes de manera crónica los efluentes de las piscifactorías marinas suponiendo un riesgo de aparición de procesos de eutrofización en el ecosistema receptor (Dosdat et al., 1995), como las proliferaciones fitoplanctónicas o el aumento de macroalgas oportunistas. Los efluentes de las piscifactorías también contienen restos de distintos tipos de biocidas, principalmente desinfectantes y antibióticos (Black, 2002; Carballeira et al., 2012a) (Carballeira et al., 2012b; Carballeira et al., 2012c), de tal forma que la

reacción de estos compuestos con la materia orgánica puede dar lugar a compuestos altamente tóxicos (eg. subproductos de desinfección) cuyos efectos son difíciles de predecir a partir del análisis químico del agua (Sarà, 2007). De este modo, para caracterizar de forma más efectiva y realista el impacto de un efluente de esta naturaleza se han de utilizar parámetros ecológicos, determinados a través de la realización de bioensayos *in situ* (trasplantes) con productores primarios (Carballeira et al., 2012c). Además, la composición corporal de los productores primarios reflejará las condiciones del agua donde hayan sido expuestos. Asimismo, (Carballeira et al., 2010) han estudiado el efecto de los efluentes procedentes de piscifactorías marinas instaladas en tierra (Land-based marine fish farms, LBMFFs) sobre la producción primaria costera mediante bioensayos realizados con discos de la macroalga *Ulva* spp.

El objetivo de este estudio es conocer, por primera vez, la dispersión y las alteraciones causadas por los efluentes de las LBMFFs sobre la comunidad fitoplanctónica nativa mediante el análisis de contenidos corporales y la medida de respuestas fisiológicas. La relación de isótopos estables ($\delta^{15}N$) ha demostrado ser un buen indicador del grado de exposición a los vertidos, los índices pigmentarios y la fluorescencia clorofílica como una evidencia del grado estrés y la concentración de clorofila *a* como una medida de la eutrofización (producción neta de biomasa de la comunidad fitoplanctónica).

2. Material y métodos

2.1. Área de estudio

Los experimentos se desarrollan en el área de influencia de dos LBMFFs (Figura 1), localizadas en la costa de Galicia (NO Península Ibérica) y dedicadas al cultivo de rodaballo (*Psetta maxima* L.) y de lenguado (*Solea solea* L.). Las piscifactorías ubicadas en las localidades de Lira y Xove producen de media 1195 y 2250 $t.año^{-1}$, respectivamente. Se han encontrado diferencias en las características físico-químicas del agua de entrada y de salida de las dos granjas estudiadas y de otras 18 instaladas en la costa gallega (Carballeira et al., 2012c).

2.2. Bioensayo de la comunidad de fitoplancton

Los bioensayos de fitoplancton fueron realizados según el método descrito por (Dalsgaard and Krause-Jensen, 2006). Para ello, se se utilizaron bolsas de diálisis en

las que se introdujeron 700ml de agua con la comunidad fitoplanctónica control. Para evitar la rotura de las frágiles bolsas de diálisis éstas se protegieron con malla de nylon y posteriormente se suspendieron de boyas náuticas ancladas al fondo y situadas a distancias crecientes del foco de vertido en cada escenario, aproximadamente a 50, 100, 200, 400 y 800m (Figura 1). En cada boya (buoy station, BS) se expusieron, a 1m de profundidad, 4 bolsas de diálisis durante 2 días. Los bioensayos se realizaron por duplicado, en septiembre y octubre del 2009.

Después del período de exposición las bolsas de diálisis fueron transportadas refrigeradas (5±1°C) y en completa oscuridad al laboratorio, dónde fueron procesadas de inmediato.

La determinación de la señal isotópica del $\delta^{15}N$ se realizó a través de una muestra compuesta obtenida al mezclar 200ml de cada bolsa de la misma boya. Las muestras compuestas fueron filtradas a presión constante por un filtro pre-pesado Whatman GF/F. Los 500ml restantes de cada bolsa fueron también filtrados a presión constante por un filtro pre-pesado de membrana de nitrocelulosa negra

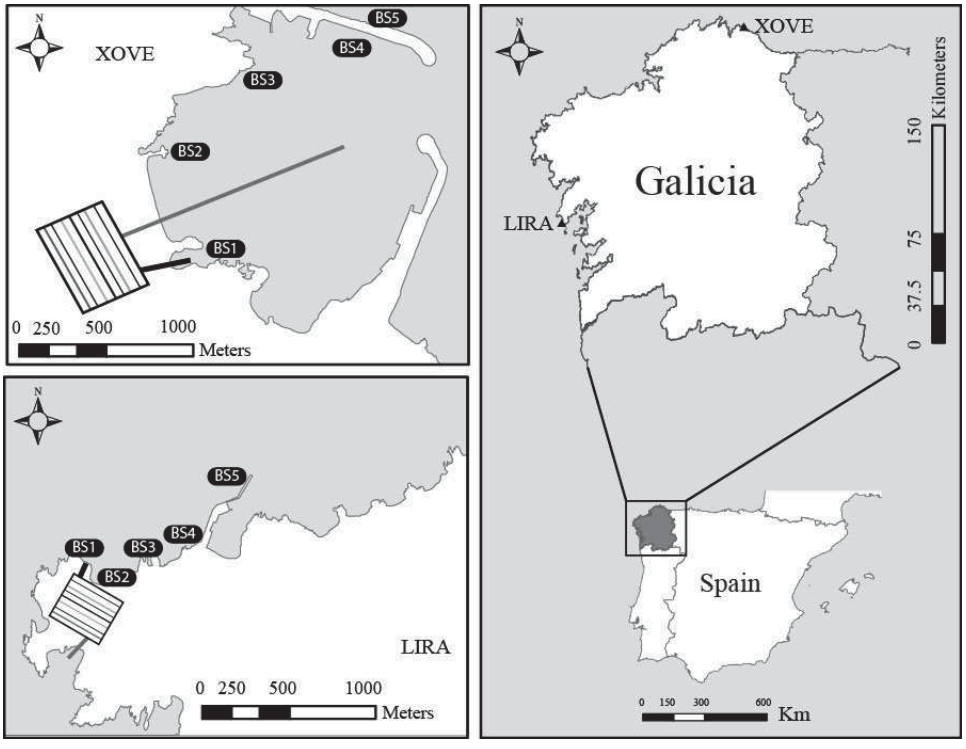


Figura 1.- Localización de las boyas (BS) en el área de influencia de dos piscifactorías marinas instaladas en tierra (Lira y Xove, Spain) donde se realizaron los experimentos.

(Black 045 μM HARP, Millipore). La fluorescencia clorofílica (ϕPSII) de la comunidad fitoplanctónica retenida en cada membrana de nitrocelulosa fue medida usando

un fluorómetro de amplitud modulada (PAM-2000 Heiz Walz GMBH, Germany). Se tomaron 5 lecturas de cada membrana. A continuación de cada membrana se extrajo el fitoplancton con 10 ml de acetona al 90%, en agitación constante, durante una hora, a 15ºC y en cámara oscura para evitar la degradación de las clorofilas. A continuación se filtró el extracto (5 µM) que fue escaneado a distintas longitudes de onda (entre 400 y 750 nm) en un espectrofotómetro (Milton Roy Spectronic 3000 Array). Del mismo modo se midió la absorbancia de una muestra acidificada (se añaden 3µl de HCl 1M sobre 3ml de extracto) (Hendry et al., 1987). Con las densidades ópticas obtenidas se calculó el índice de feofetinización D_{665}/D_{665a} y la concentración de clorofila a (*Chl a*) (Vollenweider, 1974). El mismo procedimiento analítico fue aplicado a una muestra de fitoplancton antes de cada período de exposición (t_0).

Antes de analizar la señal isotópica $\delta^{15}N$ las membranas fueron secadas a 40 ºC y alicuotas de 3 mg de muestra fueron pesadas y empaquetadas en cápsulas de estaño (EuroVector). Las cápsulas fueron almacenadas en desecador hasta el análisis de la $\delta^{15}N$, realizado por la Unidad de Técnicas Instrumentales de Análisis (Servicios de Apoyo a la Investigación, Universidad de A Coruña) según el procedimiento descrito en (Carballeira et al., 2012d).

2.3. *Análisis estadístico*

Se determinaron las diferencias significativas entre pares de BS a lo largo del gradiente de exposición para cada parámetro y escenario mediante el análisis de varianza de una vía (ANOVA), utilizando el test de comparaciones múltiples de Tukey. El grado de significación estadística establecido fue del 5% ($p<0.05$). Los análisis estadísticos fueron realizados con el software informático IBM SPSS Statistics 20.

3. Resultados y discusión

Las LBMFFs enriquecen significativamente en nutrientes las aguas que utilizan como medio de cultivo. Esto se pone en evidencia al comparar las concentraciones medias de los vertidos con los valores medios determinados en las aguas superficiales (0,5-1 m) de la misma zona de la costa gallega (INTECMAR, 2008). Mientras que el enriquecimiento en nitratos es ligero, los fosfatos se ven incrementados más de 7 veces, los nitritos más de 15 y el amonio más de 60. Los

valores medios registrados fueron: 190 μg.l^{-1} de NO_3; 4,7 μg.l^{-1} de NO_2; 9 μg.l^{-1} de NH_4; 36 μg.l^{-1} de PO_4.

En la Figura 2 se muestra como varían con la distancia al foco de vertido de las piscifactorías los valores medios (±SD) de los parámetros ensayados (*D665/D665a*, ϕPSII, *Chl a y* $\delta^{15}N$) de la comunidad de fitoplancton nativa al final del período de exposición, para los dos períodos ensayados.

Todas las BS, incluidas las consideradas como control (BS5), presentan una reducción significativa del índice de feofetinización (*D665/D665a*) respecto al valor inicial (t_0). Sin embargo, no se observan respuestas graduales claras con la distancia al foco en ninguno de los dos escenarios. Solo se observa un incremento significativo en las BS5 frente a BS4.

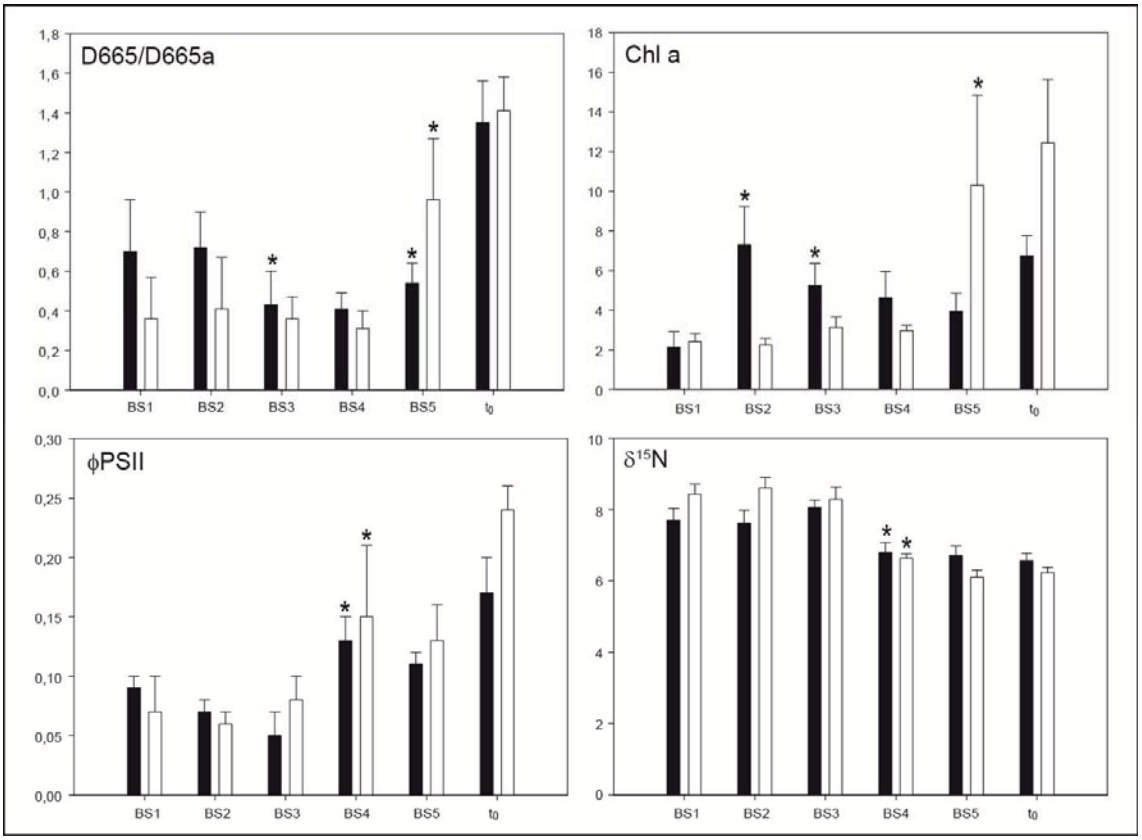


Figura 2.- Valor (media ±SD) del índice de feofetinización (D665/D665a), de la fluorescencia clorofílica (ϕPSII), de la concentración de clorofila a (Chl *a*) y de la señal isotópica ($\delta^{15}N$) de la comunidad fitoplanctónica nativa antes (t_0) y después de 2 días de exposición a los efluentes de dos LBMFFs (Lira y Xove). Para cada BS se han considerado 4 réplicas y 2 tiempos (n=8). Los valores significativos ($p<0,05$) de una BS frente a la anterior, empezando desde el foco, se han señalado con *].

Por el contrario, en ambas granjas se observa una reducción significativa de la ϕPSII en las boyas más próximas al foco (BS1, BS2 y BS3) respecto al control (BS 5) y a la

condición observada al inicio del trasplante (t_0). Asimismo, las dos estaciones control (BS5) también presentan una reducción significativa respecto al estado inicial. Esto parece indicar que existe un *efecto trasplante* derivado de la manipulación de las muestras y/o del confinamiento en las bolsas de diálisis. Este efecto puede ser causado por la reducción de la turbulencia en el interior de la bolsa y la disminución del intercambio gaseoso y de nutrientes con el exterior. De esta forma se puede enmascarar el efecto tóxico-trófico que pudieran ejercer los efluentes.

Los índices pigmentarios y, sobretodo, la fluorescencia clorofílica, sólo representan el estado del fitoplancton al final del período de incubación, mientras que la concentración de *Chl a* es una medida integradora al representar la producción neta de biomasa durante el período de incubación. Las concentraciones de *Chl a* iniciales se encuentran dentro del rango normal de variación de las aguas de estas costas en otoño (Villares and Carballeira, 2004). Los efluentes de la granja instalada en Xove inhiben significativamente la *Chl a* en todas las BS frente al control (BS5) y el período inicial (t_0). Sólo en BS5 la reducción no es significativa frente a t_0. En Lira todas las BS excepto la BS2 presentan una reducción significativa de la *Chl a* respecto a t_0. Destaca el hecho de que la concentración de *Chl a* registrada en la BS2 sea superior al control, ya que, excepto esta estación, en todos los casos la *Chl a* al final del período de incubación se encuentra por debajo del valor medio inicial.

Otros estudios (Dalsgaard and Krause-Jensen, 2006; Herbeck and Unger, *In press*) realizados con la misma metodología muestran que las jaulas marinas provocan un estímulo claro de la producción fitoplanctónica y que este bioensayo es una buena medida del riesgo de eutrofización que pueden generar sus efluentes. Sin embargo, ocurre todo lo contrario en el caso de las granjas instaladas en tierra de este estudio. La inhibición de la producción fitoplanctónica observada podría haber sido debida al "*efecto trasplante*", a la larga duración del período de incubación o a la mayor presencia de sustancias tóxicas en los efluentes de las LBMFFs.

En experiencias previas observamos, sobretodo en Xove, que incubaciones de 4 y 6 días provocaban un deterioro de las bolsas de diálisis, debido al rápido tupido generado por la deposición de material particulado, epifitismo o por procesos de descomposición de las membranas. Debido a ello, se optó por realizar incubaciones de menor duración (2 días). Los incrementos de la señal isotópica

alcanzados en las estaciones más próximas al foco indican que los procesos de asimilación de nutrientes durante este período de incubación son suficientemente intensos como para originar incrementos significativos de la producción fitoplanctónica. Como esto no se produjo los resultados obtenidos pueden atribuirse a la presencia de compuestos inhibidores o al efecto trasplante. El estrés manifestado por la relación *D665/D665a* y la ϕPSII de las estaciones consideradas control (BS5) apoyan la hipótesis de un efecto trasplante considerable. El efecto trasplante se podría minimizar aumentando el período de incubación sino aparecieran los efectos adversos anteriormente indicados. Este efecto trasplante unido a otros factores físico-químicos como la reducción del pH, el aumento de la turbidez por sólidos en suspensión o la presencia de contaminantes podrían explicar en gran parte los resultados obtenidos.

Otro aspecto a considerar es que las especies fitoplanctónicas nativas se desarrollan a una velocidad próxima a la tasa máxima de crecimiento. Sólo si se añaden nutrientes y algunas especies son desplazadas por otras especies oportunistas la producción primaria del sistema podría aumentar (Tett et al., 2007). Esto estaŕ limitado por el confinamiento de la comunidad fitoplanctónica que supone la membrana de diálisis. Así, mientras el desarrollo de las especies sensibles es inhibido por los efluentes de las LBMFFs las especies oportunistas del exterior no pueden acceder a los recursos liberados dentro de las bolsas de diálisis.

En los dos escenarios se observó un incremento significativo de la señal isotópica ($\delta^{15}N$) en las BS más cercanas al foco (BS1, BS2, BS3), mientras que las más alejadas (B4 y B5) mantienen valores próximos a los iniciales (t_0). Por varios posibles motivos ya mencionados, no se observa una relación clara entre la $\delta^{15}N$ y las respuestas biológicas de los trasplantes.

El bioensayo diseñado (Dalsgaard and Krause-Jensen, 2006) tiene un gran interés pues permite realizar un control ecotoxicológico de la calidad del agua al máximo nivel de jerarquía biológica como es la integridad ecológica de la comunidad fitoplanctónica. Es la primera vez que se utiliza este bioensayo para comprobar el efecto de los efluentes procedentes de LBMFFs. No obstante, en este caso los resultados no fueron tan evidentes como los obtenidos con el bioensayo de discos de *Ulva* spp. (Carballeira et al., 2010), aún así demostraron que existen diferencias en la calidad de los efluentes de las granjas terrestres y las jaulas marinas. A pesar de ello, igual que ocurrió en trabajos anteriores con macroalgas y moluscos

(Carballeira et al., 2012d; Carballeira et al., 2011), se confirma la utilidad de la $\delta^{15}N$ de la comunidad fitoplanctónica como indicador del área de influencia de las LBMFFs y para verificar la efectividad de las medidas tomadas para la protección ambiental.

Agradecimientos

Este estudio fue parcialmente financiado por el Plan Nacional de Acuicultura Marina. Proyecto JACUMAR (2008): "*Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)*".

Bibliografía

Black, K., Cook, E., Jones, K., Kelly, M., Leakey, R., Nickell, T., Sayer, M., Tett, P., Willis, K., 2002. Review and synthesis of the environmental impacts of aquaculture. Scottish Executive Central Research Unit.

Carballeira, C., De Orte, M.R., Viana, I.G., Carballeira, A., 2012a. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. Ecotoxicology and Environmental Safety 78, 148-161.

Carballeira, C., Espinosa, J., Carballeira, A., 2011. Linking $\delta^{15}N$ and histopathological effects in molluscs exposed *in situ* to effluents from land-based marine fish farms. Marine Pollution Bulletin 62, 2633-2641.

Carballeira, C., Orte, M.R., Viana, I.G., DelValls, T.A., Carballeira, A., 2012b. Assessing the Toxicity of Chemical Compounds Associated With Land-Based Marine Fish Farms: The Sea Urchin Embryo Bioassay With Paracentrotus lividus and Arbacia lixula. Archives of Environmental Contamination and Toxicology 63, 249-261.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, M.L., DelValls, T.A., Carballeira, A., 2012c. Designing an Integrated Environmental Monitoring Plan for Land-Based Marine Fish Farms Located at Exposed and Hard Bottom Coastal Areas. Journal of Environmental Monitoring 14, 1305-1316.

Carballeira, C., Viana, I., Carballeira, A., 2012d. δ15N values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms. Journal of Applied Phycology, 1-11.

Carballeira, C., Viana, I., Rey-Asensio, A., Carballeira, A., 2010. Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: bioensayos de fertilidad *in situ*, in: M. Rey Méndez, J.F.C., M. Izquierdo Rodriguez y A. Guerra Díaz (Ed.), XIII Foro dos Recursos Mariños e da Acuicultura das Rías Galegas. USC, O Grove, pp. 201-208.

Dalsgaard, T., Krause-Jensen, D., 2006. Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. Aquaculture 256, 302-310.

Dosdat, A., Gaumet, F., Chartois, H., 1995. Marine aquaculture effluent monitoring: Methodological approach to the evaluation of nitrogen and phosphorus excretion by fish. Aquacultural Engineering 14, 59-84.

Hendry, G.A.F., Houghton, J.D., Brown, S.B., 1987. The Degradation of Chlorophyll-A Biological Enigma. New Phytologist 107, 255-302.

Herbeck, L.S., Unger, D., In press. Dispersal and impact of aquaculture pond effluents along back-reef waters traced by nitrogen stable isotopes (^{15}N) and phytoplankton bioassays. Marine Ecology Progress Series.

INTECMAR, 2008. Anuario oceanográfico de Galicia 2008. Instituto Tecnolóxico para o Control do Medio Mariño de Galicia, Vilagarcía de Arousa.

OSPAR, C., 2003. The OSPAR Integrated Report 2003 on the Eutrophication Status of the OSPAR Maritime Area based upon the first application of the Comprehensive Procedure, Eutrophication series. OSPAR, Paris.

Sarà, G., 2007. A meta-analysis on the ecological effects of aquaculture on the water column: Dissolved nutrients. Marine Environmental Research 63, 390-408.

Tett, P., Gowen, R., Mills, D., Fernandes, T., Gilpin, L., Huxham, M., Kennington, K., Read, P., Service, M., Wilkinson, M., Malcolm, S., 2007. Defining and detecting undesirable disturbance in the context of marine eutrophication. Marine Pollution Bulletin 55, 282-297.

Villares, R., Carballeira, A., 2004. Nutrient Limitation in Macroalgae (*Ulva* and *Enteromorpha*) from the Rías Baixas (NW Spain). Marine Ecology 25, 225-243.

Vollenweider, R.A., Talling, J.F., Westlake, D.F., 1974. A manual on methods for measuring primary production in aquatic environments. Blackwell Scientific Pub.

Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioensayos *in situ* de discos de ulva

Carballeira[1], C., Rey-Asensio[2], A., Carballeira[2], A.

[1]Departamento de Química Física, Cátedra UNESCO/UNITWIN/WICOP, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain. Email: carlos.carballeira@uca.es.
[2]Grupo de Ecotoxicología, Área de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

Resumen

Son escasos los estudios sobre el impacto ambiental de los efluentes de las piscifactorías marinas instaladas en tierra (LBMFF). Los bioensayos realizados *in situ* con discos de la macroalga oportunista *Ulva* spp. (como representante de los productores primarios bentónicos) son una forma eficaz de medir el efecto tóxico y trófico de estas granjas. Los discos se expusieron a distancias crecientes del foco de vertido de dos LBMFFs y al final del período de incubación se caracterizó el efecto de los vertidos con la concentración corporal de N, la señal isotópica ($\delta^{15}N$), la concentración de clorofilas (Chl *a* y *b*), los índices pigmentarios (Chl *a*/Chl *b*, D665/D665a, D430/410), la fluorescencia clorofílica (ϕPSII) y la tasa de crecimiento relativo (RGR) en términos de incremento neto de biomasa.
No se observaron diferencias significativas en las concentraciones de clorofilas, índices pigmentarios y fluorescencia clorofílica, indicadores de ausencia de estrés en los discos trasplantados. Por el contrario, se observó que el contenido tisular de N y la señal isotópica ($\delta^{15}N$) aumentaban gradual y significativamente con el nivel de exposición a los efluentes. Los vertidos inhiben de manera significativa la RGR de los discos de ulva. La RGR disminuyó a medida que aumentaba el contenido de N y la $\delta^{15}N$ en los discos, pero sólo lo hizo de manera significativa con la $\delta^{15}N$. La $\delta^{15}N$ determinada en ulva es un buen descriptor del grado de exposición a los efluentes, informa sobre la procedencia del N y no se ve afectada ni por la regulación metabólica, ni por la existencia de saturación en su acumulación, ni por la presencia de otras sustancias tóxicas presentes en el vertido que inhiben la RGR.

Palabras clave: Aquaculture; fish farms effluents; toxicity-fertility tests; stress index; stable isotopes; eutrophication.

1. Introducción

Los residuos de las piscifactorías marinas instaladas en tierra (LBMFF) están enriquecidos en nutrientes (N y P) procedentes del metabolismo de los

peces, las heces y restos de pienso no consumido (Hall et al., 1992; Handy and Poxton, 1993; Holby, 1991; Dosdat et al., 1995), pero también contienen biocidas (desinfectantes, antibióticos, etc.) y sus respectivos derivados. La mezcla de estos compuestos puede dar lugar a efectos adversos tróficos y tóxicos en el ecosistema receptor (Black et al., 2002; Carballeira et al., 2012a; Carballeira et al., 2012b).

La monitorización de la concentración de nutrientes en la columna de agua es el método usado tradicionalmente para caracterizar el enriquecimiento nutritivo del medio marino. Sin embargo, en los ecosistemas costeros de la zona templada es muy pequeña la correlación entre la concentración de nutrientes en la columna de agua y el contenido en nutrientes de los productores primarios (Fong et al., 1998; Nedwell et al., 2002; Villares and Carballeira, 2006). Esto es debido a que en el proceso trófico intervienen muchos factores que interaccionan de manera compleja condicionando la disponibilidad de la carga de nutrientes (Cloern, 2001; Martins and Marques, 2002; Aldridge and Trimmer, 2009). Si a esto le añadimos las confusas interacciones que se pueden generar con la presencia de sustancias tóxicas se puede concluir que es muy arriesgado evaluar el riesgo que supone un vertido en función de la concentración de los elementos que transporta. Por otro lado, la vigilancia ambiental basada en la analítica del agua es onerosa debido a que las concentraciones varían fuertemente a nivel diario/horario lo cual requiere un esfuerzo de muestreo intensivo y extensivo (Karakassis et al., 2001). Aun así, la información de la columna de agua puede considerarse parcialmente defectuosa e insuficiente para explicar los posibles efectos de la acuicultura sobre los procesos ecológicos (Sarà, 2007). Además, la rápida dilución y retirada biológica de nutrientes también dificulta la detección de los contaminantes con las técnicas analíticas convencionales. En consecuencia, para evaluar el impacto de un efluente es preferible utilizar un parámetro de respuesta biológico que medir un elevado número de variables físico-químicas en el agua.

Una vía de diagnóstico efectiva para conocer el potencial trófico/tóxico de un vertido consiste en la realización de bioensayos con productores primarios. Los protocolos de los bioensayos con algas están bien desarrollados y, además, estos organismos permiten realizar trasplantes con

facilidad (Lyngby and Mortensen, 1994; Hernández et al., 1997; Costanzo et al., 2001; An, 2003; Dalsgaard and Krause-Jensen, 2006).

Las respuestas de los productores primarios expuestos a los contaminantes *in situ* suponen una guía realista de caracterización del riesgo porque integran la acción conjunta de todos los factores abióticos y bióticos que intervienen en el proceso. Además, el tiempo de renovación comparativamente largo de los tejidos de las plantas en condiciones de campo suministra una imagen integrada y persistente de la disponibilidad de nutrientes aunque la aportación se produjese de manera esporádica.

Los isótopos estables pueden ser usados como trazadores de nutrientes permitiendo caracterizar su origen mediante valores específicos (Costanzo et al., 2001). Numerosos estudios han empleado la señal isotópica $\delta^{15}N$ para investigar el impacto potencial de los efluentes de diferente naturaleza, incluidos los procedentes de las piscifactorías marinas instaladas en jaulas o en tierra (Jones et al., 2001; Thimdee et al., 2002; Vizzini and Mazzola, 2004; Costanzo et al., 2004; Sará et al., 2006; Pérez et al., 2008; Holmer and Kristensen, 1992; Carballeira et al., 2012c; Carballeira et al., 2011; García-Sanz et al., 2010; García-Sanz et al., 2011).

Su aplicabilidad se basa en que el N procedente de las granjas marinas presenta una alta proporción de ^{15}N frente al nivel de referencia del N inorgánico marino, como resultado de la volatilización del amonio rico en ^{15}N y del procesado microbiano del N en disolución (Van Dover et al., 1992). El valor de la $\delta^{15}N$ en macroalgas puede ser especialmente eficaz como trazador de las fuentes de nutrientes, porque estas especies no fraccionan ni seleccionan los isótopos de nitrógeno a lo largo de un amplio rango de concentraciones y ratios isotópicos (Cohen and Fong, 2005). Además, los bioensayos con macroalgas, dispuestos a modo de gradiente respecto al foco de emisión, son herramientas eficaces de control porque permiten caracterizar de manera precisa la extensión e intensidad del impacto generado por el efluente (Carballeira et al., 2012c; Costanzo et al., 2001; Dalsgaard and Krause-Jensen, 2006; Deutsch and Voss, 2006; García-Sanz et al., 2010; García-Sanz et al., 2011).

El objetivo de este estudio es evaluar, por primera vez, la dispersión y el impacto de los efluentes de las LBMFFs sobre la producción primaria costera

mediante el bioensayo *in situ* de discos de macroalgas. Se seleccionó *Ulva* spp., por ser el alga con mayor presencia en la mareas verdes de las costas atlánticas europeas. Además de controlar la tasa de crecimiento relativo (RGR) de los discos de ulva expuestos a los efluentes se pretende estudiar en qué medida los contenidos corporales (N, $\delta^{15}N$ y clorofilas) y las respuestas fisiológicas (índices pigmentarios, fluorescencia clorofílica) explican los efectos observados sobre la producción primaria y puedan ser usados como indicadores del impacto observado.

2. Material y métodos

2.1. Área de estudio

Los experimentos se desarrollaron en el área de influencia de dos LBMFF, localizadas en la costa de Galicia (NW Spain), dedicadas al cultivo de rodaballo (*Psetta maxima* L., 1758) y ocasionalmente de lenguado (*Solea solea* L., 1758). Las LBMFF, ubicadas en las localidades de Lira y Xove, producen de media 1195 y 2250 t/año, respectivamente (Figura 1). La agencia medioambiental responsable de estas granjas (Augas de Galicia, XUGA) suministra los valores físico-químicas del agua procedente de los

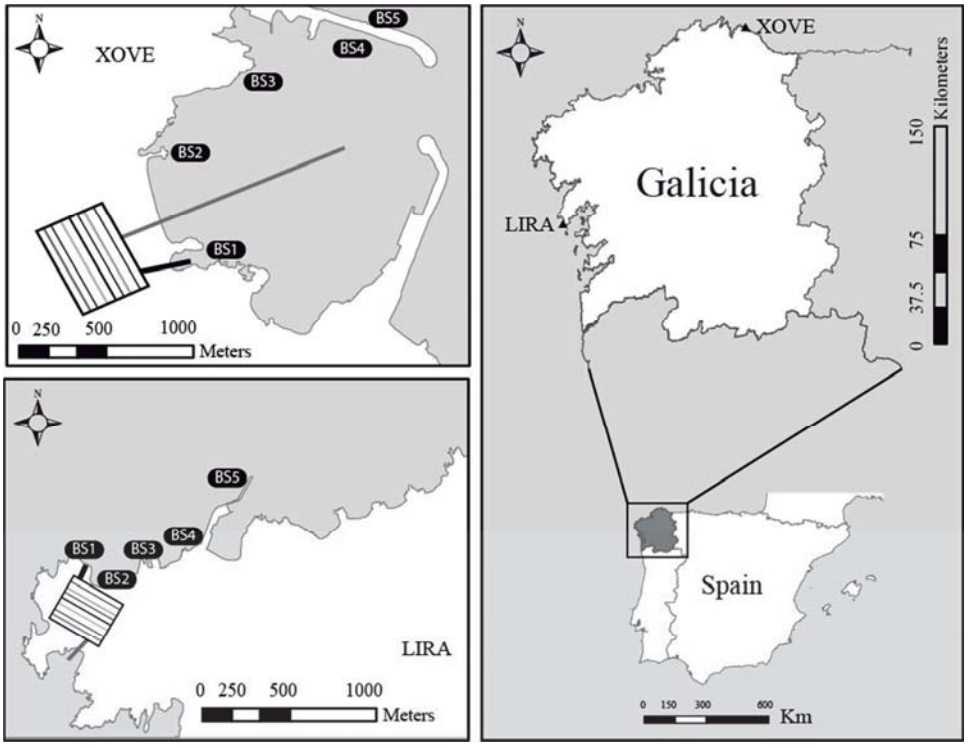


Figura 1.- Localización de las boyas (BS) en el área de influencia de dos piscifactorías marinas instaladas en tierra (Lira y Xove, Spain) donde se realizaron los experimentos.

controles de verificación de los vertidos de las LBMFFs, tanto del agua de entrada como de salida (Tabla I).

Adicionalmente, se realizaron mediciones físico-químicas del agua de salida de las dos granjas de estudio para comprobar que estos valores estaban dentro de la media de las granjas (Tabla I).

2.2. Bioensayo de discos de ulva

Los bioensayos de discos de *Ulva* spp. fueron realizados según el método descrito por Dalsgaard and Krause-Jensen (2006). Se recolectaron frondes de *Ulva* en una zona control unas pocas horas antes de realizar los trasplantes. Inmediatamente, se extrajeron los discos de algas (∅1,4 cm) con un sacabocados de plástico de la región media del talo. Durante el procesado tanto los frondes como los discos se mantuvieron en neveras portátiles y oscuridad a 5±1 ºC.

Tabla 1. Características físico-químicas de los efluentes muestreados en dos piscifactorías marinas instaladas en tierra (LBMFF) en la costa de Galicia (Lira y Xove) y el valor medio de las características físico-químicas de los efluentes muestreados por Augas de Galicia en 18 LBMFF durante el periodo 2002-08. [Los valores de Salinidad, pH, Oxigeno, Sólidos en suspensión (SS), Carbono orgánico total (TOC), Fosfatos, Nitritos, Nitratos y Amonio. SD=Desviación estándar; n= número de análisis; ±Δ = output-input].

LBMFF	Salinidad g L^{-1}	pH	O_2 mg L^{-1}	O_2 %	SS mg L^{-1}	TOC mg L^{-1}	PO_4 mg L^{-1}	NO_2 mg L^{-1}	NO_3 mg L^{-1}	NH_3 mg L^{-1}
Lira	34,7	7,63	7,8	79,1	21	8,04	0,29	0,080	0,205	0,78
Xove	32,4	7,48	8,3	79,6	23	5,64	0,22	0,086	0,190	0,85
Media	34,5	7,7	8,3	84,2	18	7,63	0,24	0,075	0,198	0,56
± SD	0,4	0,61	0,2	4,6	12	1,94	0,04	0,016	0,022	0,42
n	179	179	162	162	179	103	143	179	24	19
±Δ salida-entrada	0,2	-0,3	-0,2	-1,7	5	1,58	0,06	0,034	0,028	0,25

Tras la extracción de los discos de la fronde principal se produce la activación de la esporulación por los bordes en un porcentaje alto de los discos, los cuales sufren una decoloración pigmentaria y a continuación pueden llegar a deshacerse y desaparecer (Salomonsen et al., 1999). Este fenómeno, conocido como discos fantasma ("ghost tissue"), reduce el

número de discos al final del período de exposición lo cual disminuye la fiabilidad del ensayo. Para evitar la aparición de discos fantasmas se tratan con una disolución de 0.5 ml/l NaClO durante 60 segundos (Viana et al., 2009). A continuación, los discos se colocaron en cámaras de metacrilato cilíndricas (∅ 10 x 20 cm) cerradas lateralmente con una malla de fibra de vidrio (luz de malla 2mm). Las cámaras se suspendieron de boyas náuticas ancladas al fondo (BS) y situadas a distancias crecientes respecto al foco del vertido. El gradiente de exposición en Lira se estableció con las siguientes distancias al foco: 50, 100, 200, 400 y 800 m. En el caso de Xove se localizaron en gradiente todas las BS dentro de la zona portuaria excepto la considerada control (Figura 1). En cada BS se dispusieron a 1,5 m de profundidad y durante 6 días, 4 cámaras con 25 discos de *Ulva* en cada cámara. Los bioensayos se realizaron por duplicado.

Los bioensayos fueron realizados en el período de máxima producción de las LBMFF (Septiembre-Octubre 2008) con el objeto de reflejar el mayor impacto posible.

2.3. Análisis químico y biológico

Después de cada período de exposición (*t*), las cámaras son transportadas al laboratorio en nevera (5±1°C) donde se realizan inmediatamente las medidas de estrés fisiológico.

2.3.1. Fluorescencia clorofílica

La eficiencia fotosintética fue evaluada mediante la fluorescencia clorofílica registrada sobre la superficie del disco de *Ulva* spp. Las medidas de fluorescencia clorofílica fueron tomadas para una densidad de flujo fotónico fotosintético (PPFD) de 1400 µmol m^{-2} s^{-1}, usando un fluorómetro portátil de pulsos de amplitud modulada (Photosynthesis Yield Analyzer Fluorometer, Heiz Walz GMBH, Germany). La eficiencia cuántica del fotosistema II (ϕPSII) representa la eficiencia del fotosistema II en la captura de la luz fotoquímica (Genty et al., 1989). Se realizaron 6 lecturas con el fin de minimizar la variabilidad entre discos de ulva.

2.3.2. Análisis de los pigmentos fotosintéticos

Para la extracción de los pigmentos se tomaron 50-60 mg (peso fresco) de los discos de ulva de cada cámara que fueron machacados en mortero de ágata y extraídos con acetona al 90% durante 1 h en oscuridad (Vollenweider et al., 1974). El extracto fue centrifugado a 3000 rpm durante 3 min. y posteriormente se realizaron lecturas de las absorbancias entre 400 y 750 nm, en un espectrofotómetro (Milton Roy Spectronic 3000 Array). Cada extracto fue acidificado con 20 ml de 1 N HCl y a continuación se volvieron a leer las absorbancias.

Las concentraciones de clorofila a (Chla) y b (Chlb) fueron calculadas con la fórmula propuesta por (Vollenweider et al., 1974).. Además, se calcularon las relaciones e índices clorofílicos: *Chl a/b*, *Chl a+b*, D_{665}/D_{665a} y D_{430}/D_{410}. Cada muestra fue analizada por duplicado. El mismo procedimiento analítico fue aplicado a una muestra de los discos antes del período de trasplante (t_0).

2.3.3. Tasa relativa de crecimiento (RGR)

Para la determinación del incremento de biomasa se seleccionaron 10 discos de ulva enteros y se secaron a 40 °C en una estufa de tiro forzado (P-Selecta Digitronic) hasta alcanzar un peso constante. Se entiende como tasa relativa de crecimiento (RGR) la relación:

$$RGR = \frac{B_t - B_{t_0}}{t}$$

Donde B_0 y B_t son la biomasa del disco en el tiempo cero (t_0) y después del período de exposición *t* (6 días), respectivamente.

2.3.4. Análisis de nitrógeno total (N) y del isótopo estable ($\delta^{15}N$)

Para la determinación del N total y de la señal isotópica $\delta^{15}N$ los discos de ulva secos fueron pulverizados y homogeneizados utilizando un molino orbital de bolas (Retsch PM 100). Alícuotas (≈ 3 mg) de las muestras fueron pesadas y empaquetadas en cápsulas de estaño (EuroVector). Las capsulas fueron almacenadas en desecador hasta el análisis del N y $\delta^{15}N$, realizado

en la Unidad de Técnicas Instrumentales de Análisis (UTIA), Servicios de Apoyo a la Investigación de la Universidad de A Coruña. Las muestras fueron quemadas en un analizador elemental (FlashEA1112: ThermoFinnigan) acoplado a un espectrofotómetro de masas (Deltaplus: ThermoFinnigan). La acetanilida fue usada como material de referencia estándar para cuantificar el contenido de nitrógeno. La calibración para el gas de referencia (N_2) fue llevado a cabo con IAEA-N-1 ($(NH_4)2SO_4$), IAEA-N-2 ($(NH_4)2SO_4$) y IAEA-NO-3 (KNO_3) como patrones estándar. La abundancia relativa de ^{15}N en las muestras ($\delta^{15}N$) fue calculada como: $\delta^{15}N$ (‰) = $[(R_{sample}/R_{standard})-1]\times10^3$, donde R es la relación $^{15}N/^{14}N$ y $R_{standard}$ la composición del aire (Robinson, 2001). El error global determinado con el uso de réplicas analíticas (n=9) fue del 8%.

2.4. Análisis estadístico

Las diferencias estadísticamente significativas entre las boyas fueron analizadas por el test no paramétrico Kruskal–Wallis con post hoc (Zar, 1996), mediante el paquete estadístico SPSS versión 21.0. Las correlaciones lineares entre variables se comprobaron con el coeficiente de correlación de Pearson. La hipótesis nula fue rechazada, en todos los casos, con un 5% de significación.

3. Resultados

Las LBMFF enriquecen significativamente en nutrientes las aguas que utilizan. Esto se pone en evidencia si comparamos las concentraciones medias de los vertidos (Tabla 1) con los valores medios determinados en las aguas superficiales (0,5-1m) de la misma costa de Galicia (INTECMAR, 2008). Mientras que el enriquecimiento en nitratos es ligero, los fosfatos se ven incrementados más de 7 veces, los nitritos más de 15 y el amonio más de 60. Los valores medios registrados fueron: 190 $\mu g.l^{-1}$ de NO_3; 4,7 $\mu g.l^{-1}$ de NO_2; 9 $\mu g.l^{-1}$ de NH_4; 36 $\mu g.l^{-1}$ de PO_4.

3.1. Medidas de estrés fisiológico

En la Figura 2 se muestra como varía los valores medios (± SD) el contenido en clorofilas (*Chl a, Chl b, Chl a+b*), los índices pigmentarios (*Chl a/Chl b*,

D665/D665a, D430/D410) y la fluorescencia clorofílica (ϕPSII) con la distancia al foco del vertido de las granjas (Lira y Xove) para los dos tiempos ensayados. Ninguno de estos parámetros presentó diferencias significativas entre las cinco boyas (BS) de Lira. En Xove se observaron diferencias significativas (P<0,05) entre las BS para las ratio *Chl a/Chl b* y *D665/D665a*, pero en ningún caso se observó una respuesta gradual con la distancia al foco. La eficiencia cuántica del fotosistema (ϕPSII) y las ratio entre clorofilas de los discos expuestos se consideran valores normales en *Ulva* sp. (ϕPSII ~ 0,7; *Chl a/Chl b* ~ 1,7) (Xia et al., 2004).

La disminución en la actividad puede estar relacionada con cambios en el contenido de clorofila. Una reducción en la clorofila, ya sea debido a una disminución en la biosíntesis o aumento de la tasa de degradación, es un síntoma de toxicidad común de las algas (Gledhill et al., 1997). Sin embargo, en ninguno de los escenarios se observaron diferencias significativas en el contenido clorofílico. En experimentos de corta duración y altos niveles de nutrientes la ulva no presentó diferencias significativas en el contenido de la *Chl a* (Figueroa et al., 2008).

La fluorescencia clorofílica es un indicador del estado fisiológico del alga frente a un período de estrés. La fluorescencia clorofílica ha sido previamente usada como monitor sensible de estrés fisiológico en condiciones de campo (Cabello-Pasini et al., 2000) y para controlar el crecimiento de macroalgas nativas y de cultivo, incluida la *Ulva* spp., bajo la acción de efluentes de piscifactorías en estanque de *Sparus aurata* (Figueroa et al., 2006; Figueroa et al., 2008).

Aunque no se observaron diferencias significativas en la fluorescencia clorofílica entre las BS de cada escenario, el comportamiento fue diferente en las granjas. En Lira los menores valores se observan cerca del foco de vertido (B1 y B2) y aumentan con la distancia (B3 y B4) superando al control (B5). Por el contrario, en Xove la fluorescencia aumenta con la exposición a los vertidos registrando el menor valor el control (B5).

La falta de una respuesta gradual con la distancia al foco y la ausencia de diferencias significativas entre las cinco boyas (BS) de cada escenario de los parámetros de estrés indican que los efluentes de las LBMFFs parecen no afectar ni a la acumulación de pigmentos fotosintéticos ni al proceso de la

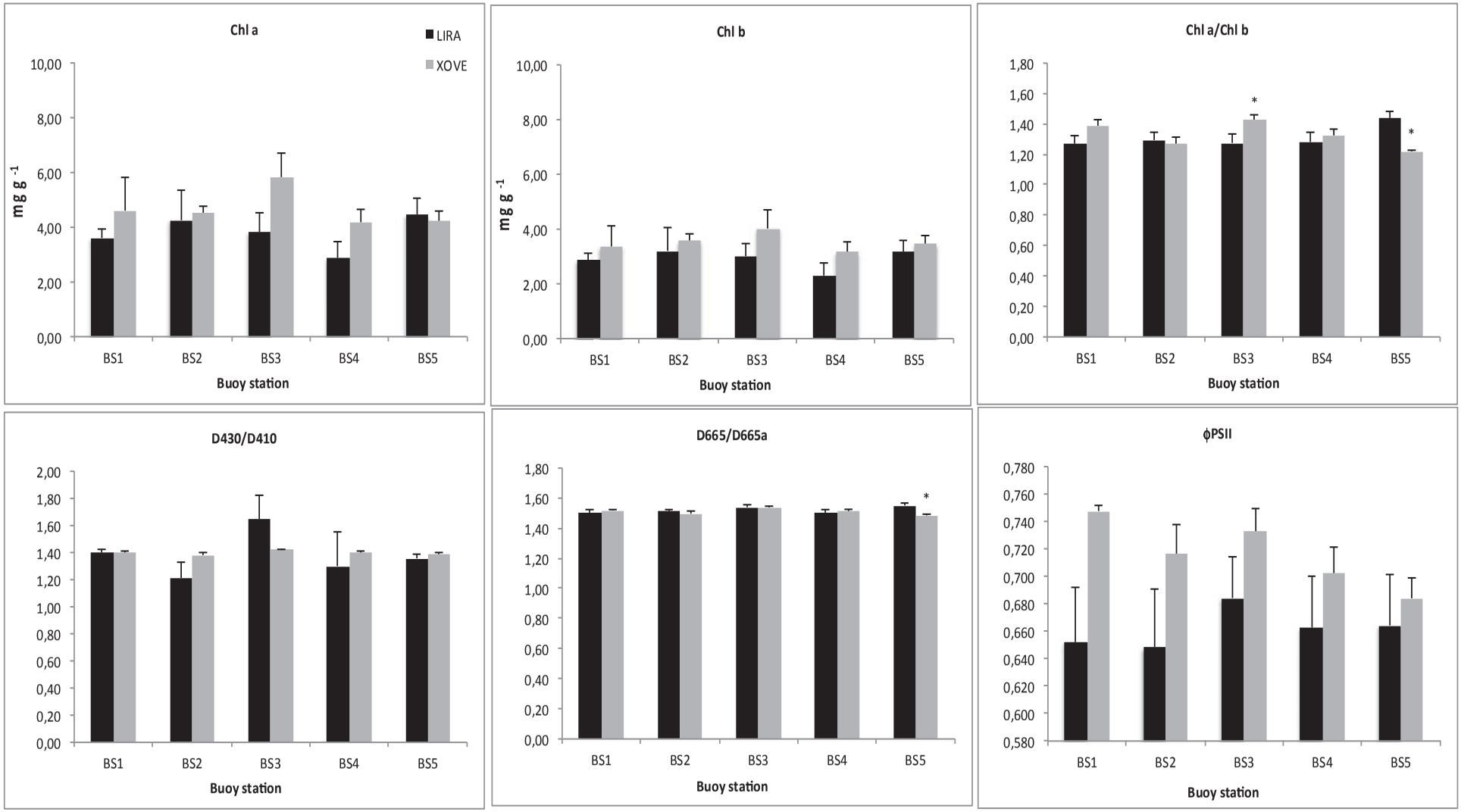


Figura 2. Variación (media±SD), con la distancia al foco de vertido de las granjas (Lira y Xove), del contenido en clorofilas (*Chl a y b*), de los índices pigmentarios (*Chl a/Chl b, D665/D665a, D430/D410*) y de la fluorescencia clorofílica (ϕPSII) determinada en los discos de *Ulva* sp al final del período de exposición (t). [*: valor significativo (P<0,05) en una BS frente a la BS anterior, empezando por el foco].

fotosíntesis. Esto puede ser debido a que las especies de *Ulva* son consideradas generalmente muy tolerantes al efecto de los contaminantes en comparación con otras macroalgas (Haritonidis and Malea, 1999; Lee and Wang, 2001).

3.2. Tasa relativa de crecimiento (RGR)

El incremento medio de biomasa de los discos durante el período de exposición fluctuó entre 4 y 24 mg dw.day^{-1}. En Xove la tasa relativa de crecimiento (RGR) tiende a aumentar con la distancia al foco. Los mayores valores y significativamente diferentes (P<0,001) del resto se dan en la estación control localizada fuera del puerto, fuera de la influencia de las LBMFF. En este caso, el efecto inhibidor de los efluentes es superior al efecto estimulante de la carga de nutrientes. Los efluentes de Xove inhiben significativamente la RGR respecto al control pero no existen diferencias significativas entre las BS localizadas dentro del área portuaria.

Los efluentes de Lira inhiben la RGR, que aumentan con la distancia con la significativa excepción de la estación control.

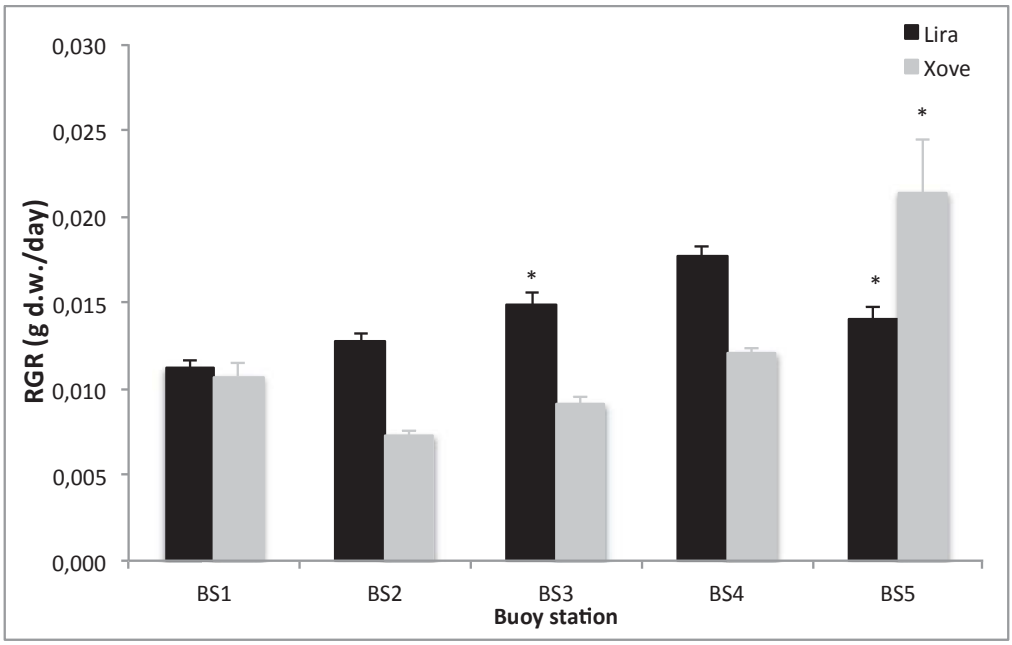


Figura 3. Variación de la tasa relativa de crecimiento (RGR),con la distancia al foco de vertido de las dos granjas (Lira y Xove), de los discos de *Ulva* sp, expresada como incremento medio de biomasa seca diaria (g_{dw}.day^{-1}) durante el período de exposición. [*: valor significativo (P<0,05) en una BS frente a la BS anterior, empezando por el foco].

3.3. Análisis de los isótopos estables ($\delta^{15}N$) y del nitrógeno total (N)

El contenido en N de los discos de *Ulva* al final del período de exposición disminuye significativamente con la distancia al foco en Xove, mientras que no se encontraron diferencias significativas entre las BS de Lira. Esto puede ser debido a que como la tasa de renovación hídrica es limitada en la zona portuaria de Xove los trasplantes responden claramente a la carga de N de los efluentes de la LBMFF.

La señal isotópica, tiende a disminuir gradual y significativamente con la distancia al foco de vertido en ambos escenarios. Los discos de la macroalga trasplantada presentaron en el t_0 un valor de $\delta^{15}N$ elevado (8,3±0,6‰), de ahí los porcentajes negativos encontrados al final del periodo de exposición, sobre todo en las BS más alejadas del foco (Figura 3). A pesar de la alta señal isotópica inicial las dos estaciones consideradas control (BS5), al final del período de exposición recuperan valores próximos al nivel de fondo regional ($\delta^{15}N$ ≈6,4 ‰) (Viana et al., 2011). El efecto de los efluentes se hace notar claramente en toda la zona portuaria de Xove y en Lira hasta >400m de distancia a pesar de ser una zona con un elevado hidrodinamismo. Usando esta técnica se llegó a detectar incrementos de la $\delta^{15}N$ en macroalgas a >1 km distancia de piscifactorías en jaulas instaladas en el Mediterráneo y en el Atlántico (García-Sanz et al., 2010; García-Sanz et al., 2011).

A diferencia de los otros parámetros se observaron diferencias significativas entre las BS de cada granja tanto para N total y $\delta^{15}N$ (Figura 4) como para las RGR de los discos de la macroalga (Figura 3). Esto puede ser debido, a que el contenido en N y sobretodo de la $\delta^{15}N$ y la RGR integran la situación ambiental ocurrida durante todo el período de exposición.

3.4. Relaciones entre variables

Se estudió la relación entre el contenido clorofílico (Chl *a+b*), la fluorescencia clorofílica, el N tisular y la $\delta^{15}N$ frente a la RGR de los discos de *Ulva* sp trasplantados (Tabla 2). La fluorescencia clorofílica y la concentración de clorofilas (Chl *a+b*) pueden ser relacionadas con la RGR como medidas de la tasa fotosintética o la eficiencia pigmentaria, respectivamente (Arntz et al., 2000; Villares and Carballeira, 2004). Sin

embargo, en ningún caso (Tabla 2) se observó una correlación significativa entre estos parámetros y la RGR. La falta de correlación entre la tasa fotosintética y la RGR puede ser debido a que la primera es una medida puntual al final del período de exposición y la segunda es el producto de un

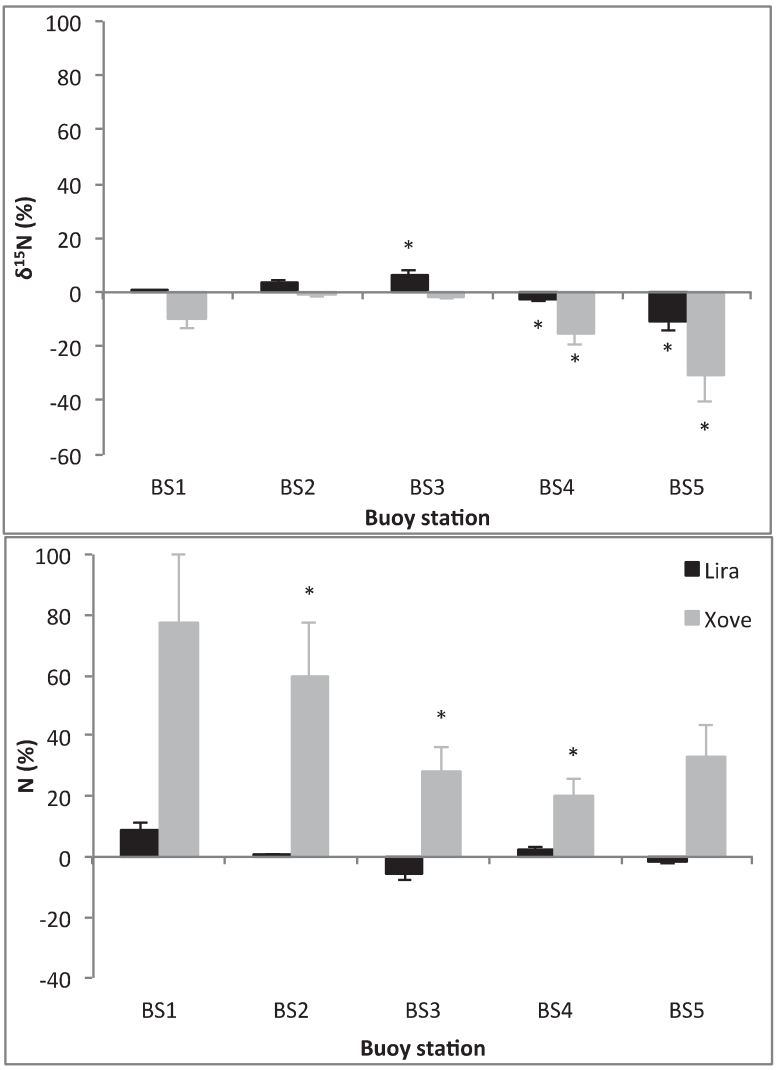


Figura 4. Variación media (%) del contenido de N y de la δ ^{15}N en los discos de *Ulva* sp. trasplantados en las boyas (BS) en dos escenarios (Lira y Xove) al final del período de exposición (t) respecto al momento inicial (t_0) [$(t-t_0)/t_0).100$)]. [*: valor significativo ($p<0,05$) en una BS frente a la BS anterior, empezando por el foco].

proceso acumulativo durante todo el período. Además, no necesariamente una alta tasa fotosintética se asocia necesariamente a una alta tasa de crecimiento por que pueden intervenir otros factores (Arntz et al., 2000).

En la Tabla 2 se puede observar como en todos los casos, tratando los datos de las dos LBMFF por separado o en conjunto, el N y la δ^{15}N se correlacionan negativamente con la RGR, pero sólo son significativas para la δ^{15}N. Cuando se analizan los datos de cada granja por separado, sólo se obtienen

correlaciones significativas en Xove. Destaca la correlación con $\delta^{15}N$ observada en Xove (R^2=0,86) y también promediando el tiempo de exposición para las dos granjas (R^2=0,49).

4. Discusión

El factor más importante asociado a los procesos de eutrofización marina es la excesiva carga de nutrientes de origen terrestre que baña las áreas costeras. De forma general, el nitrógeno es considerado el factor limitante del crecimiento algal en las aguas marinas costeras (Ryther and Dunstan, 1971; Morand and Briand, 1996). En este sentido, la liberación de nutrientes (fundamentalmente de N) por las LBMFFs puede causar problemas ambientales y, por ello, el análisis de la concentración de nutrientes en los vertidos o en el entorno es la técnica habitual de control ambiental.

Sin embargo, habitualmente los parámetros de calidad del agua fluctúan significativamente en cortos períodos de tiempo al ser dispersados y diluidos rápidamente por las corrientes (Wolanski et al., 2000). Esto complica la cuantificación mediante técnicas analíticas estándar ya que obliga tomar un gran número de muestras lo que aumenta el coste de la vigilancia ambiental (Karakassis et al., 2001; Dalsgaard and Krause-Jensen, 2006). Además, los altos caudales bombeados por las LBMFF (Tabla 1) diluyen los nutrientes de tal forma que los incrementos específicos son difícilmente detectables. A todo esto hay que añadir la falta de modelos capaces de predecir el riesgo ambiental con la información suministrada por dichos análisis. Solamente en condiciones de alta carga y baja capacidad dispersiva del medio se puede medir en el efluente el enriquecimiento de alguno de los parámetros clásicos, como el amonio en disolución que puede ser útil para obtener una aproximación del riesgo trófico (Porrello et al., 2003).

En el caso de las LBMFF los nutrientes se descargan muy diluidos pero al ser emitidos de manera crónica son captados constantemente por los productores primarios de los alrededores. La concentración de nutrientes en los tejidos de las macroalgas puede ser utilizado, frente los análisis tradicionales del agua, como un indicador de las condiciones del agua en que se han desarrollado (Pedersen and Borum, 1996). El método se basa en

comparar el contenido en nutrientes del biomonitor con sus cuotas de subsistencia y concentraciones críticas (Ho, 1987; Wheeler and Björnsäter, 2004; Fong et al., 1998; Lyngby and Mortensen, 1994; Lyngby et al., 1999; Villares et al., 1999; Villares and Carballeira, 2006).

Sin embargo, este método presenta ciertos inconvenientes ya que los niveles de referencia deben ser tomados con precaución. Las concentraciones críticas de los nutrientes pueden estar afectadas por diversos factores como la luz u otros nutrientes, y pueden variar estacionalmente (Fujita et al., 1989; Lobban and Harrison, 1994; Figueroa et al., 2008; Figueroa et al., 2006) no encontraron diferencias significativas en el contenido de N en individuos de *Ulva* spp. que crecen bajo la influencia de efluentes piscícolas. La situación normal en la costa donde se realizaron los experimentos consistiría en que los valores de N en agua y en *Ulva* spp. fuesen altos en invierno y bajos en verano (Villares and Carballeira, 2006). Esto apoya la realización de los bioensayos de fertilidad en el período de máxima producción de las granjas (verano-otoño) dado que es cuando los efluentes más pueden estimular el crecimiento natural del alga por ser el momento en que se producen las mayores emisiones de nutrientes y cuando el medio natural puede ser más limitante. Aun así, los valores de N en los discos trasplantados se encontraron siempre desde el inicio (22,7±0,6 $mg.gdw^{-1}$) hasta el final del período de exposición (25,2±2,1$mg.gdw^{-1}$) muy por encima de la cuota de subsistencia (8,13 $mg.gdw^{-1}$) y fueron muy similares a las concentraciones críticas (24,5$mg.gdw^{-1}$) estimadas para la *Ulva* spp que habita en las costas de Galicia (Villares and Carballeira, 2004). Por ello, se considera que este método solamente es útil cuando las algas se encuentran en condiciones limitantes de nutrientes extremas. Una vía efectiva para determinar el estado nutritivo de una macroalga es estudiar la relación entre la composición corporal y el crecimiento (Björnsäter and Wheeler, 1990). Sin embargo, la falta de correlación (Tabla 2) entre el contenido de N y la RGR nos lleva a considerar que el bioensayo de macroalgas basado en la medida del crecimiento en función de la distancia al foco del vertido sería el método más adecuado en la evaluación del riesgo (Lyngby and Mortensen, 1994).

Tabla 2. Coeficientes de correlación (R^2) y significación estadística (* p<0,05, **p< 0,01) entre la $\delta^{15}N$ (‰), N tisular (%), *Chl a+b* ($mg.g^{-1}$) y ϕPSII frente a la tasa de crecimiento relativo (RGR= a ± b X) de los discos de *Ulva* spp. expuestos a los vertidos de las LBMFFs.

LBMFF	**Datos**		**$\delta^{15}N$ (‰0)**			**N (%)**			**Chl a+b ($mg.g^{-1}$)**				**(ϕPSII)**		
	F= Farm T=Times	n	a	b	R^2	a	b	R^2	a	b	R^2	a	b	R^2
LIRA+XOVE	2F and 2T	20	0,0320	-0,0022	0,210*	0,0200	-0,0026	0,067	0,0166	-0,0004	0,069	0,0247	-0,0165	0,072
LIRA+XOVE	2F	10	0,0420	-0,0034	0,490**	0,0260	-0,0049	0,112	0,0236	-0,0014	0,175	0,0472	-0,0492	0,181
LIRA	1F and 2T	10	0,0157	-0,0002	0,002	0,0127	0,0006	0,011	0,0158	-0,0007	0,034	0,0164	-0,0035	0,017
XOVE	1F and 2T	10	0,0356	-0,0028	0,330*	0,0233	-0,0038	0,072	0,0160	-0,0001	0,057	0,0689	-0,0788	0,247
LIRA	1F	5	0,0225	-0,0319	0,048	0,0319	-0,0075	0,161	0,0242	-0,0006	0,369	0,0818	-0,0401	0,224
XOVE	1F	5	0,0487	-0,0043	0,863**	0,0296	-0,0060	0,194	0,0260	-0,0006	0,091	0,1175	-0,1466	0,476

Otra alternativa es utilizar un descriptor de exposición como la $\delta^{15}N$. (Lin and Fong, 2008) utilizaron el crecimiento, el N tisular y la $\delta^{15}N$ en macroalgas para detectar el efecto originado por una granja de langostinos y comprueban que el más sensible de estos indicadores es la señal isotópica.

Las RGR obtenidas (0,012-0,027 $g_{dw}.day^{-1}$) se encuentran dentro del rango observado en *Ulva*. La RGR son muy bajas al principio del verano, siendo la tasa media mínima en junio de 0,017 $g_{dw}.day^{-1}$, que se va incrementando hacia el otoño donde se pueden registrar valores puntuales de 0,072 $g_{dw}.day^{-1}$ en Noviembre (Villares and Carballeira, 2006).

Dalsgaard and Krause-Jensen (2006) observaron, en discos de *Ulva* trasplantados, que aumentaba claramente el contenido de N, la concentración de Chl a y la RGR con la proximidad a las granjas de peces en jaulas. Sin embargo, esto no ocurre así en los discos expuestos a los vertidos de las LBMFFs, cuya RGR, en términos de biomasa, puede ser inhibida o estimulada dependiendo de la intensidad de la exposición, que es descrita por la distancia al foco y por la $\delta^{15}N$, mejor que por el contenido de N (Tabla 2).

El N tisular puede disminuir con la distancia al foco, como se observó en Xove o mantenerse constante como ocurrió en Lira. Debido a la gran heterogeneidad de las condiciones nutritivas que se hallan en las áreas costeras –sobre todo en la zona intermareal- hace que el contenido corporal de nutrientes no sea siempre un indicador válido de procesos de eutrofización (Villares and Carballeira, 2006).

Por otro lado, la RGR del alga puede estar condicionada por factores físico-químicos como la turbidez, pH o la presencia de contaminantes. Así, la disminución del pH (Tabla 1) entre el agua de entrada y salida, es un indicador de la presencia de contaminantes (residuos metabólicos, antibióticos, desinfectantes, etc.) y puede afectar a la asimilación del N amoniacal (Carballeira et al., 2012c). Las correlaciones negativas entre la RGR y la $\delta^{15}N$, como medida del grado de exposición a los vertidos, indican que los efectos tóxicos de los vertidos de las LBMFF se pueden superponer a los potenciales efectos tróficos. Sólo cuando se alcanza un determinado grado de dilución de los contaminantes se puede producir la activación del crecimiento. De esta manera, en la Figura 4 se puede observar como en Lira

la mayor RGR se produce a 400m (BS 4) del foco, que es superior a la del control (BS 5) localizado a 800m.

La naturaleza de las comunidades de fondos rocosos está determinada por las relaciones de los distintos miembros de la comunidad y la dinámica de la comunidad está fuertemente influenciada por los organismos formadores de hábitat, como las macrófitas (Jones and Andrew, 1992; Jones et al., 1994). Cualquier proceso que pueda influir sobre los organismos formadores de hábitat a menudo conlleva efectos cascada sobre el resto de los organismos que componen la comunidad. Como los organismos fotosintéticos sésiles responden fielmente a la alteración de los factores ambientales, bióticos y abióticos, pueden ser utilizados como bioindicadores sensibles de esos cambios (Orfanidis et al., 2001, 2003). Entre las medidas que mejor pueden caracterizar un impacto ambiental está la productividad macrofitobentónica, pero el coste y la complejidad de la medida *in situ* limita su aplicación en la vigilancia ambiental. Por este motivo, los bioensayos realizados *in situ* con discos de *Ulva* trasplantados son una alternativa poco onerosa y los resultados indican si los vertidos de las LBMFFs pueden afectar significativamente a la RGR de las macroalgas. Esto puede crear un desequilibrio de los ecosistemas instalados en las zonas mareal y submareal cuando son inhibidos o estimulados por los efluentes de las piscifactorías.

5. Conclusiones

Por primera vez, se utilizan trasplantes de discos de *Ulva* para evaluar el riesgo ecotoxicológico de los vertidos de las LBMFFs de forma sencilla, realista y anticipada a la respuesta del ecosistema. Se ha comprobado que, según el grado de exposición y el correspondiente equilibrio tóxico-trófico, los vertidos pueden inhibir o estimular significativamente la producción primaria pudiendo perturbar el correcto funcionamiento del ecosistema receptor. Si las LBMFFs se localizan en zonas dispersivas la rápida dilución de los efluentes hace que el efecto sea menor y no se extienda más de 400m.

La $\delta^{15}N$ en macroalgas es una herramienta de detección temprana útil para la vigilancia ambiental de este tipo de vertidos y que permite anticiparse al deterioro ambiental. Además, el similar comportamiento de las diferentes

especies de macroalgas frente a la $\delta^{15}N$ facilita su aplicación como indicador de impacto a escala local.

La correlación significativa entre la señal isotópica ($\delta^{15}N$) y la RGR determinada en los discos de *ulva* permite detectar las zonas "calientes" (distancia hasta donde se altera significativamente la actividad de los productores primarios) o verificar la efectividad de las medidas tomadas para la protección ambiental.

Agradecimientos

Este estudio fue parcialmente financiado por el Plan Nacional de Acuicultura Marina. Proyecto JACUMAR (2008): "*Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)*".

Bibliografía

Aldridge, J., Trimmer, M., 2009. Modelling the distribution and growth of 'problem' green seaweed in the Medway estuary, UK. Hydrobiologia 629, 107-122.

An, K.-G., 2003. Spatial and Temporal Variabilities of Nutrient Limitation Based on *In Situ* Experiments of Nutrient Enrichment Bioassay. Journal of Environmental Science and Health, Part A 38, 867-882.

Arntz, A.M., DeLucia, E.H., Jordan, N., 2000. From fluorescence to fitness: variation in photosynthetic rate affects fecundity and survivorship. Ecology 81, 2567-2576.

Björnsäter, B.R., Wheeler, P.A., 1990. Effect of nitrogen and phosphorus supply on growth and tissue composition of *Ulva fenestrata* and *Enteromorpha intestinalis* (ulvales, chlorophyta). J. Phycol. 26, 603-611.

Black, K., Cook, E., Jones, K., Kelly, M., Leakey, R., Nickell, T., Sayer, M., Tett, P., Willis, K., 2002. Review and synthesis of the environmental impacts of aquaculture. Report for the Scottish Executive Central Research Unit.

Cabello-Pasini, A., Aguirre-von-Wobeser, E., Figueroa, F.L., 2000. Photoinhibition of photosynthesis in *Macrocystis pyrifera* (Phaeophyceae), *Chondrus crispus* (Rhodophyceae) and *Ulva lactuca* (Chlorophyceae) in outdoor culture systems. Journal of Photochemistry and Photobiology B: Biology 57, 169-178.

Carballeira, C., De Orte, M.R., Viana, I.G., Carballeira, A., 2012a. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. Ecotoxicol. Environ. Saf. 78, 148-161.

Carballeira, C., Espinosa, J., Carballeira, A., 2011. Linking $\delta^{15}N$ and histopathological effects in molluscs exposed *in situ* to effluents from land-based marine fish farms. Mar. Pollut. Bull. 62, 2633-2641.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, M.L., DelValls, T.A., Carballeira, A., 2012b. Designing an Integrated Environmental Monitoring Plan for Land-Based Marine Fish Farms Located at Exposed and Hard Bottom Coastal Areas. J. Environ. Monit. 14, 1305-1316.

Carballeira, C., Viana, I., Carballeira, A., 2012c. $\delta^{15}N$ values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms. J. Appl. Phycol., 1-11.

Cloern, J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. Mar. Ecol. Prog. Ser. 210, 223-253.

Cohen, R.A., Fong, P., 2005. Experimental evidence supports the use of $\delta^{15}N$ content of the opportunistic green macroalga *Enteromorpha intestinalis* (chlorophyta) to determine nitrogen sources to estuaries. J. Phycol. 41, 287-293.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., 2004. Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. Mar. Pollut. Bull. 48, 514-525.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., Loneragan, N.R., Thomas, M., 2001. A New Approach for Detecting and Mapping Sewage Impacts. Mar. Pollut. Bull. 42, 149-156.

Dalsgaard, T., Krause-Jensen, D., 2006. Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. Aquaculture 256, 302-310.

Deutsch, B., Voss, M., 2006. Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: Results from *in-situ* incubation experiments. Sci. Total Environ. 366, 799-808.

Dosdat, A., Gaumet, F., Chartois, H., 1995. Marine aquaculture effluent monitoring: Methodological approach to the evaluation of nitrogen and phosphorus excretion by fish. Aquac. Eng. 14, 59-84.

Figueroa, F.L., Bueno, A., Korbee, N., Santos, R., Mata, L., Schuenhoff, A., 2008. Accumulation of Mycosporine-like Amino Acids in *Asparagopsis armata* Grown in Tanks with Fishpond Effluents of Gilthead Sea Bream, *Sparus aurata*. J. World Aquacult. Soc. 39, 692-699.

Figueroa, F.L., Santos, R., Conde-Álvarez, R., Mata, L., Gómez Pinchetti, J.L., Matos, J., Huovinen, P., Schuenhoff, A., Silva, J., 2006. The use of chlorophyll fluorescence for monitoring photosynthetic condition of two tank-cultivated red macroalgae using fishpond effluents, Bot. Mar., 275.

Fong, P., Boyer, K.E., Zedler, J.B., 1998. Developing an indicator of nutrient enrichment in coastal estuaries and lagoons using tissue nitrogen content of the opportunistic alga, *Enteromorpha intestinalis*. J. Exp. Mar. Biol. Ecol. 231, 63-79.

Fujita, R.M., Wheeler, P.A., Edwards, R.L., 1989. Assessment of macroalgal nitrogen limitation in a seasonal upwelling region.

García-Sanz, T., Ruiz-Fernández, J.M., Ruiz, M., García, R., González, M.N., Pérez, M., 2010. An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. Mar. Environ. Res. 70, 189-200.

García-Sanz, T., Ruiz, J.M., Pérez, M., Ruiz, M., 2011. Assessment of dissolved nutrients dispersal derived from offshore fish-farm using nitrogen stable isotope ratios ($\delta^{15}N$) in macroalgal bioassays. Estuarine, Coastal and Shelf Science 91, 361-370.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) - General Subjects 990, 87-92.

Gledhill, M., Nimmo, M., Hill, S.J., Brown, M.T., 1997. The toxicity of copper (ii) species to marine algae, with particular reference to macroalgae. J. Phycol. 33, 2-11.

Hall, P.O.J., Holby, O., Kollberg, S., Samuelsson, M.O., 1992. Chemical fluxes and mass balances in a marine fish cage farm. 4. Nitrogen. Marine ecology progress series. Oldendorf 89, 81-91.

Handy, R.D., Poxton, M.G., 1993. Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. Rev. Fish Biol. Fish. 3, 205-241.

Haritonidis, S., Malea, P., 1999. Bioaccumulation of metals by the green alga *Ulva rigida* from Thermaikos Gulf, Greece. Environ. Pollut. 104, 365-372.

Hernández, I., Peralta, G., Pérez-Lloréns, J.L., Vergara, J.J., Niell, F.X., 1997. Biomass and dynamics of growth of *Ulva* species in Palmones river estuary. J. Phycol. 33, 764-772.

Ho, Y., 1987. *Ulva lactuca*(Chlorophyta, Ulvales) in Hong Kong intertidal waters-- its nitrogen and phosphorus contents and its use as a bioindicator of eutrophication. Asian Mar. Biol. 4, 97-102.

Holby, O., 1991. Chemical flux and mass balances in a marine fish cage farm. II. Phosphorus. Mar. Ecol. Prog. Ser. 70, 263-272.

Holmer, M., Kristensen, E., 1992. Impact of marine fish cage farming on metabolism and sulfate reduction of underlying sediments. Marine Ecology Progress Series MESEDT 80.

INTECMAR, 2008. Anuario oceanográfico de Galicia 2008. Instituto Tecnolóxico para o Control do Medio Mariño de Galicia, Vilagarcía de Arousa.

Jones, A.B., O'Donohue, M.J., Udy, J., Dennison, W.C., 2001. Assessing Ecological Impacts of Shrimp and Sewage Effluent: Biological Indicators with Standard Water Quality Analyses. Estuarine, Coastal and Shelf Science 52, 91-109.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. Oikos, 373-386.

Jones, G., Andrew, N., 1992. Temperate reefs and the scope of seascape ecology, Proceedings of the Second International Temperate Reef Symposium.

Karakassis, I., Tsapakis, M., Hatziyanni, E., Pitta, P., 2001. Diel variation of nutrients and chlorophyll in sea bream and sea bass cages in the Mediterranean. Fresenius Environmental Bulletin 10, 278-283.

Lee, W.Y., Wang, W.X., 2001. Metal accumulation in the green macroalga *Ulva fasciata* effects of nitrate, ammonium and phosphate. Sci. Total Environ. 278, 11-22.

Lin, D.T., Fong, P., 2008. Macroalgal bioindicators (growth, tissue N, $\delta^{15}N$) detect nutrient enrichment from shrimp farm effluent entering Opunohu Bay, Moorea, French Polynesia. Mar. Pollut. Bull. 56, 245-249.

Lobban, C.S., Harrison, P.J., 1994. Seaweed ecology and physiology. Press Syndicate of the University of Cambridge, Cambridge.

Lyngby, J.E., Mortensen, S., Ahrensberg, N., 1999. Bioassessment Techniques for Monitoring of Eutrophication and Nutrient Limitation in Coastal Ecosystems. Mar. Pollut. Bull. 39, 212-223.

Lyngby, J.E., Mortensen, S.M., 1994. Assessment of nutrient availability and limitation using macroalgae. Journal of Aquatic Ecosystem Stress and Recovery (Formerly Journal of Aquatic Ecosystem Health) 3, 27-34.

Martins, I., Marques, J., 2002. A Model for the Growth of Opportunistic Macroalgae (*Enteromorpha* sp.) in Tidal Estuaries. Estuarine, Coastal and Shelf Science 55, 247-257.

Morand, P., Briand, X., 1996. Excessive growth of macroalgae: a symptom of environmental disturbance. Bot. Mar. 39, 491-516.

Nedwell, D., Dong, L., Sage, A., Underwood, G., 2002. Variations of the nutrients loads to the mainland UK estuaries: correlation with catchment areas, urbanization and coastal eutrophication. Estuarine, Coastal and Shelf Science 54, 951-970.

Orfanidis, S., Panayotidis, P., Stamatis, N., 2001. Ecological evaluation of transitional and coastal waters: a marine benthic macrophytes-based model. Mediterr. Mar. Sci. 2, 45-65.

Orfanidis, S., Panayotidis, P., Stamatis, N., 2003. An insight to the ecological evaluation index (EEI). Ecological Indicators 3, 27-33.

Pedersen, M.F., Borum, J., 1996. Nutrient control of algal growth in estuarine waters. Nutrient limitation and the importance of nitrogen requirements and nitrogen storage among phytoplankton and species of macroalgae. Marine ecology progress series. Oldendorf 142, 261-272.

Pérez, M., García, T., Invers, O., Ruiz, J.M., 2008. Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish farm impact. Mar. Pollut. Bull. 56, 869-879.

Porrello, S., Lenzi, M., Tomassetti, P., Persia, E., Finoia, M.G., Mercatali, I., 2003. Reduction of aquaculture wastewater eutrophication by phytotreatment ponds system. II. Nitrogen and phosphorus content in macroalgae and sediment. Elsevier, Amsterdam.

Robinson, D., 2001. $\delta^{15}N$ as an integrator of the nitrogen cycle. Trends Ecol. Evol. 16, 153-162.

Ryther, J.H., Dunstan, W.M., 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science (New York, NY) 171, 1008.

Salomonsen, J., Flindt, M., Geertz-Hansen, O., Johansen, C., 1999. Modelling advective transport of *Ulva lactuca* (L) in the sheltered bay, Møllekrogen, Roskilde Fjord, Denmark. Hydrobiologia 397, 241-252.

Sarà, G., 2007. A meta-analysis on the ecological effects of aquaculture on the water column: Dissolved nutrients. Mar. Environ. Res. 63, 390-408.

Sará, G., Scilipoti, D., Milazzo, M., Modica, A., 2006. Use of stable isotopes to investigate dispersal of waste from fish farms as a function of hydrodynamics. Inter-Research, Oldendorf.

Thimdee, W., Deein, G., Thimdee, W., Sangrungruang, C., Matsunaga, K., 2002. High % N and $\delta^{13}C$ values in mangrove leaves and sediments of a mangrove-fringed estuary, Thailand-effect of shrimp pond effluents. Bulletin of the Society of Sea Water Science, Japan 56, 166-173.

Van Dover, C.L., Grassle, J.F., Fry, B., Garritt, R.H., Starczak, V.R., 1992. Stable isotope evidence for entry of sewage-derived organic material into a deep-sea food web. Nature 360, 153-156.

Viana, I.G., Fernández, J.A., Aboal, J.R., Carballeira, A., 2011. Measurement of $\delta^{15}N$ in macroalgae stored in an environmental specimen bank for regional scale monitoring of eutrophication in coastal areas. Ecological Indicators 11, 888-895.

Viana, I.G., Rey-Asensio, A., Carballeira, A., 2009. Eliminación de discos fantasmas en bioensayos con *Ulva* sp., in: U.d. Lisboa (Ed.), XVII Simposio Ibérico de Botánica Criptogámica. Universidade de Lisboa, Tomar, 97-98.

Villares, R., Carballeira, A., 2004. Nutrient Limitation in Macroalgae (*Ulva* and *Enteromorpha*) from the Rías Baixas (NW Spain). Mar. Ecol. 25, 225-243.

Villares, R., Carballeira, A., 2006. Trophic categorization in the Rías Baixas (NW Spain): nutrients in water and in macroalgae. Sci. Mar. 70, 89-97.

Villares, R., Puente, X., Carballeira, A., 1999. Nitrogen and phosphorus in *Ulva* sp. in the Galician Rias Bajas (northwest Spain): Seasonal fluctuations and influence on growth. Bol. Inst. Esp. Oceanogr. 15, 337-341.

Vizzini, S., Mazzola, A., 2004. Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Mar. Pollut. Bull. 49, 61-70.

Vollenweider, R.A., Talling, J.F., Westlake, D.F., 1974. A manual on methods for measuring primary production in aquatic environments. Blackwell Scientific Pub.

Wheeler, P.A., Björnsäter, B.R., 2004. Seasonal fluctuations in tissue nitrogen, phosphorus, and N: P for five macroalgal species common to the pacific northwest coast. J. Phycol. 28, 1-6.

Wolanski, E., Spagnol, S., Thomas, S., Moore, K., Alongi, D., Trott, L., Davidson, A., 2000. Modelling and visualizing the fate of shrimp pond effluent in a mangrove-fringed tidal creek. Estuarine, Coastal and Shelf Science 50, 85-97.

Xia, J., Li, Y., Zou, D., 2004. Effects of salinity stress on PSII in *Ulva lactuca* as probed by chlorophyll fluorescence measurements. Aquat. Bot. 80, 129-137.

Zar, J., 1996. Biostatistical analysis, 3 ed. Prentice Hall, Michigan.

Monitoring chronic effects of land-based marine fish farm effluents at exposed and rocky shores by using biochemical biomarkers in native mussels

C. Carballeira[1], J. Ramos-Gómez[2], M. L. Martín-Díaz[1,3], T. A. DelValls[1]

[1]UNITWIN/UNESCO/WiCoP, Departamento de Química Física, Universidad de Cádiz, Facultad de Ciencias del Mar y Ambientales, Campus Universitario de Puerto Real, 11510 Puerto Real, Cádiz, Spain

[2]Ecotoxicología, Departamento de Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, A Coruña, Spain

[3]Centro Andaluz de Ciencia y Tecnología Marinas (CACYTMAR), Universidad de Cádiz, Campus Universitario de Puerto Real, 11510 Puerto Real, Cádiz, Spain

Abstract

Little is known about the effects that effluents from land-based marine fish farms (LBMFFs) have on aquatic systems. The strong hydrodynamic conditions and rockiness that characterize coasts where LBMFFs are usually located make sediment and water chemical analyses unworkable or uneffective. Hence, different tools to assess and monitor LBMFF environmental effects are required. In the present study, a battery of biochemical biomarkers was evaluated as a possible tool for assessing the extent of contamination and the potential environmental impact of land-based marine aquaculture.

Biotransformation and antioxidant enzymes, and parameters of oxidative damage were measured in native mussels, *Mytilus galloprovincialis*, along spatial gradients starting from the discharge outlets of seven LBMFFs in Galicia (NW Spain). The activities of biotransformation and antioxidant enzymes were lowest in the gills of mussels sampled close to the farm outlet at most scenarios, suggesting inhibition or impairment of enzymatic activities. Lipid peroxidation (LPO) levels in mussels in the vicinity of the discharge outlets were high, indicating pro-oxidative stress conditions. Enzymatic activities appeared to increase, and LPO levels to decrease with increasing distance from the outlets.

Keywords: Ethoxyresorufin O-deethylase (EROD); Dibenzylfluorescein dealkylase (DBF); Glutathione-S-transferase (GST); Glutathione reductase (GR); Glutathione peroxidase (GPX); *Mytilus galloprovincialis;* aquaculture; toxicity assessment.

1. Introduction

Aquaculture has undergone a major expansion in the last few decades, in some cases farming has completely replaced fishing of some species. The increase in aquaculture activities has led to a need to evaluate and monitor the impact associated with fish farm wastes, which are known to include feed

and faeces of the grown organisms, as well as detergents, disinfectants, antifoulants, pesticides and veterinary medicines used to treat fish and clean the facilities (Read and Fernandes, 2003). The majority of the studies addressing water, sediment and biota alterations caused by marine aquaculture have focused on cage-based facilities and extensive land mariculture (Aguado-Giménez et al., 2007; Borja et al., 2009; Kullman et al., 2007; Rodríguez-Gallego et al., 2008, Tovar et al., 2000). On the contrary, research on the influence of intensive land-based marine aquaculture on aquatic systems is scarce, and there is an evident need to develop methods to assess and control the environmental impact of this industry.

Evaluation and monitoring of the environmental impact of wastes originated in intensive land-based marine fish farms (LBMFFs) can be performed according to two approaches, not mutually exclusive: the physico-chemical approach and the toxicity-based approach.

The physico-chemical approach comprises the determination of water and sediment quality parameters and the concentration of contaminants in water, sediment and organisms. These measures may help to determine the extent of contamination, the distribution of target contaminants and possible causes of biota alteration. However, when applied to evaluate the effects of intensive LBMFFs the physico-chemical approach presents a number of limitations:

i) The lack of information about the use of chemicals (Carballeira et al., 2012b). As Zitko (2001) suggested, and according to our experience of the last years, the aquaculture industry is secretive with this issue (Crane et al., 2007; Carballeira et al., 2012c). There are not records on the usage of chemical products available to the public, nor regulations that oblidge this industry to control chemical use and be transparent with the consumers (Crane et al., 2007). In consequence, researchers facing chemical monitoring of aquaculture activities must invest resources in analysing a wide range of substances (which may or may not be present in the effluent), and even so, part of the chemicals involved and of concern may be overlooked.

ii) LBMFFs are located at highly exposed coasts. The usual absence of sediment in this type of areas limits the options to study environmental matrices (Carballeira et al., 2012c). Besides, the hydrodinamic conditions and subsequent dilution and dispersion of contaminants lead to low concentrations

of contaminants in the water column even nearby the LBMFF discharge point (Carballeira et al., 2012c). This complicates the analytical procedure and reduces the precision of the measurements (Zitko, 2001), ultimately decreasing the efficacy of such analysis. It also may mislead by underestimating the effect of the aquaculture operations in the aquatic system.

iii) Chemical analysis do not provide information on the detrimental effects (Pandard et al., 2006). Although toxicological pathways of a great number of known contaminants have been established (Wright and Welbourg, 2002), predicting toxic effects by using chemical results from environmental matrices characterized by complex mixtures of contaminants is likely to be speculative and unrealistic, because not all chemicals can be identified, they may be undergoing degradation processes, and interactions amongst compounds may occur (Backhaus et al., 2003).

Because of all these limitations, the environmental surveillance of intensive LBMFFs should be based mainly on a toxicity-based approach. In many situations, it is more important to determine the impact than to characterise the chemical contaminants (Duke and Mount, 1991). Toxicity tests inform about the bioavailable and metabolically active fraction of pollutants, and they integrate the effects of all the existing contaminants — including those which have been identified and those which remain unknown— and their interactions. In fact, they have been recognized as the best method to assess environmental risk of wastes, including sewage and dredged materials, and to evaluate water and sediment quality (Pandard et al., 2006; Peters et al., 2001, Wah Chu and Chow, 2002).

Bioassays involving the study of biological alterations caused by environmental stress at high levels of biological organization (population, community or ecosystem levels) may be too complex so as to be considered as tools for routine use in monitoring plans (Moore et al., 2004). On the contrary, at the biochemical level, biomarkers have been shown to respond rapidly and sensitively to chemical contamination. Biochemical biomarkers provide an integrated response to contamination by multi-xenobiotics (Binelli et al., 2006) and allow to distinguish different levels and types of pollution (Abrahamson et al., 2007; Binelli et al., 2006; Peters and Livingstone, 2001; Pereira et al., 2011; Thibaut and Porte, 2008), and to assess the bioavailability of contaminants

(Bocchetti et al., 2008a). Besides, these biomarkers are precursors of damage at higher levels of biological organization: histological, physiological, population and community (Ramos-Gómez et al., 2011). Thus, they can be used as proactive tools to predict and prevent further and possibly irreversible consequences of environmental contamination. Biochemical biomarkers measured in aquatic organisms have been used to assess environmental pollution (Cajaraville et al., 2000; Handy et al., 2003), have already been included in several monitoring plans (Roose et al., 2011) and have recently been proposed for application in Water Framework Directive monitoring programmes (Sanchez and Porcher, 2009; Van der Oost, 2011).

The present study aimed to help fill the existing gap in the knowledge of the potential effects that intensive LBMFFs may have on a wide variety of molecular biomarkers.

The specific objectives of the study were as follows:

1) To quantify a battery of biochemical biomarkers in native specimens of the mussel *Mytilus galloprovincialis* (Lamarck, 1819) collected in the vicinity of seven LBMFFs located on the Galician Coast (NW Spain), therefore, chronically exposed to farm effluents. The activities of the biotransformation enzymes ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF) and glutathione S-transferase (GST), the activities of the antioxidant enzymes glutathione reductase (GR) and glutathione peroxidase (GPX), and the oxidative effects lipid peroxidation (LPO) and DNA strand breaks were measured.

2) To evaluate contamination exposure and the extent of the impact of waste water discharged from the LBMFFs. For this purpose, variations in biomarker responses were assessed in mussels sampled along a non linear spatial gradient starting at the discharge outlet and following the direction of the prevailing current.

3) To evaluate the validity of the biomarker approach and the sensitivity of *Mytilus galloprovincialis* as a sentinel organism for LMBFF monitoring.

2. Materials and methods

2.1. Description of the study areas

The study areas were established close to seven turbot (*Psetta maxima*) intensive LBMFFs located at rocky and highly exposed coasts in Galicia (NW Spain): farms I, II, III, IV, V, VI and VII (Fig. 1). Farm I is located in the inner zone of a bay, where it obtains the water used in the aquaculture facilities and where it also discharges the waste produced on the farm. There is also an aluminium factory in the area, and two harbours, one used for loading and unloading the factory materials and the other is a small fishing dock. The port infrastructures limit renewal of the bay water. The other six fish farms are located on exposed rocky coasts subjected to strong hydrodynamic conditions. No other sources of contamination apart from the fish farms were identified.

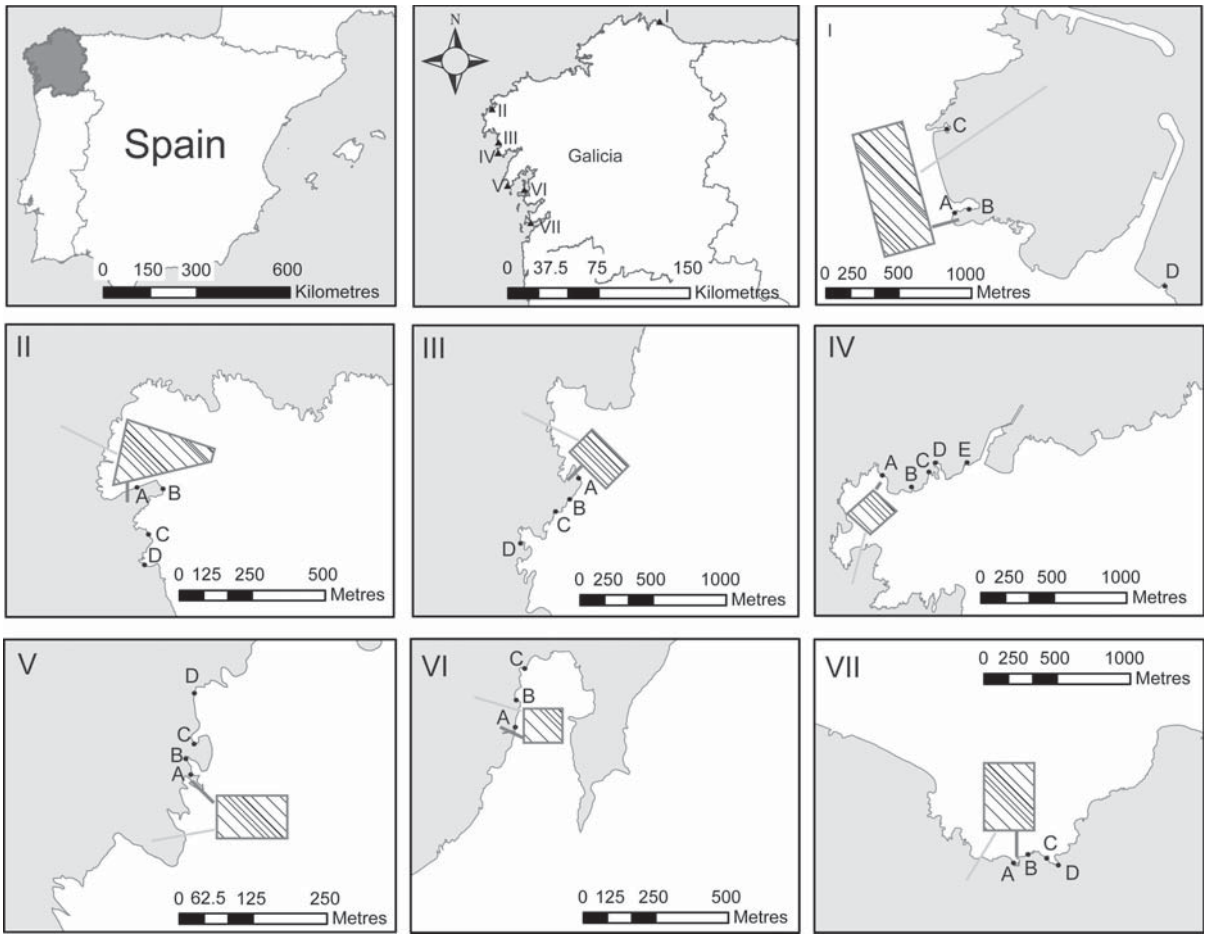


Figure 1. Map of the study areas. A: Map of Galicia with the location of the LBMFFs selected for this work. B: Location of the study sites in the different scenarios, from farm I to farm VII.

2.2. Effluent characterization

The Galician agency responsible for the environmental monitoring of the LBMFFs (Augas de Galicia) provided the physicochemical characterization of

the effluents from the seven fish farms, which comprised effluent salinity, pH, dissolved oxygen, suspended solids, total organic carbon, phosphates, nitrites, nitrates and ammonia.

2.3. Collection of biological samples

Three to five sampling sites were established in each study area, along a spatial gradient starting at the LBMFF discharge outlet and following the direction of the prevailing current (Fig. 1). Sampling sites were established according to the hydrodynamic conditions, the coastline and the fish farm production, approximately at 0, 50, 100, 200, 400 and 1000 m from the effluent, some distances varied depending on the presence of native mussels. In October 2008, ten individual specimens (5 cm shell length) of the mussel *Mytilus galloprovincialis* were collected at each sampling site, at evenly spaced intervals within a 15 m diameter area. The specimens were placed in cool boxes and transported to the laboratory.

2.4. Processing of samples and biomarker analysis

The mussels were dissected *in vivo*. Gills and hepatopancreas were removed and stored individually at -80ºC until analysis.

Previously to the analysis, samples were thawed and maintained at 4ºC during the subsequent processing. Pairs of gills and hepatopancreas samples were combined to provide pooled tissue samples. These were then weighed and homogenized in an Ultra-Turrax® homogenizer at a ratio of 1:4 (sample weight : buffer volume). The homogenization buffer contained 100 mM NaCl, 25 mM HEPES, 0.1 mM EDTA and 0.1 mM dithiotheritol.

Some of the homogenate was assigned for total protein, LPO and DNA strand breaks determinations. The remainder was centrifuged (15000 g, 4ºC, 20 min) in order to obtain the S_{15} fraction. The S_{15} supernatant was used to analyze total proteins, and EROD, DBF, GST, GR and GPX activities.

The analytical procedures for biomarker determinations were adaptations of different methods. These adaptations have been validated by Martín-Díaz et al. (2008, 2009) and Ramos-Gómez et al. (2011). The summarized methodologies and references are shown in Table 1.

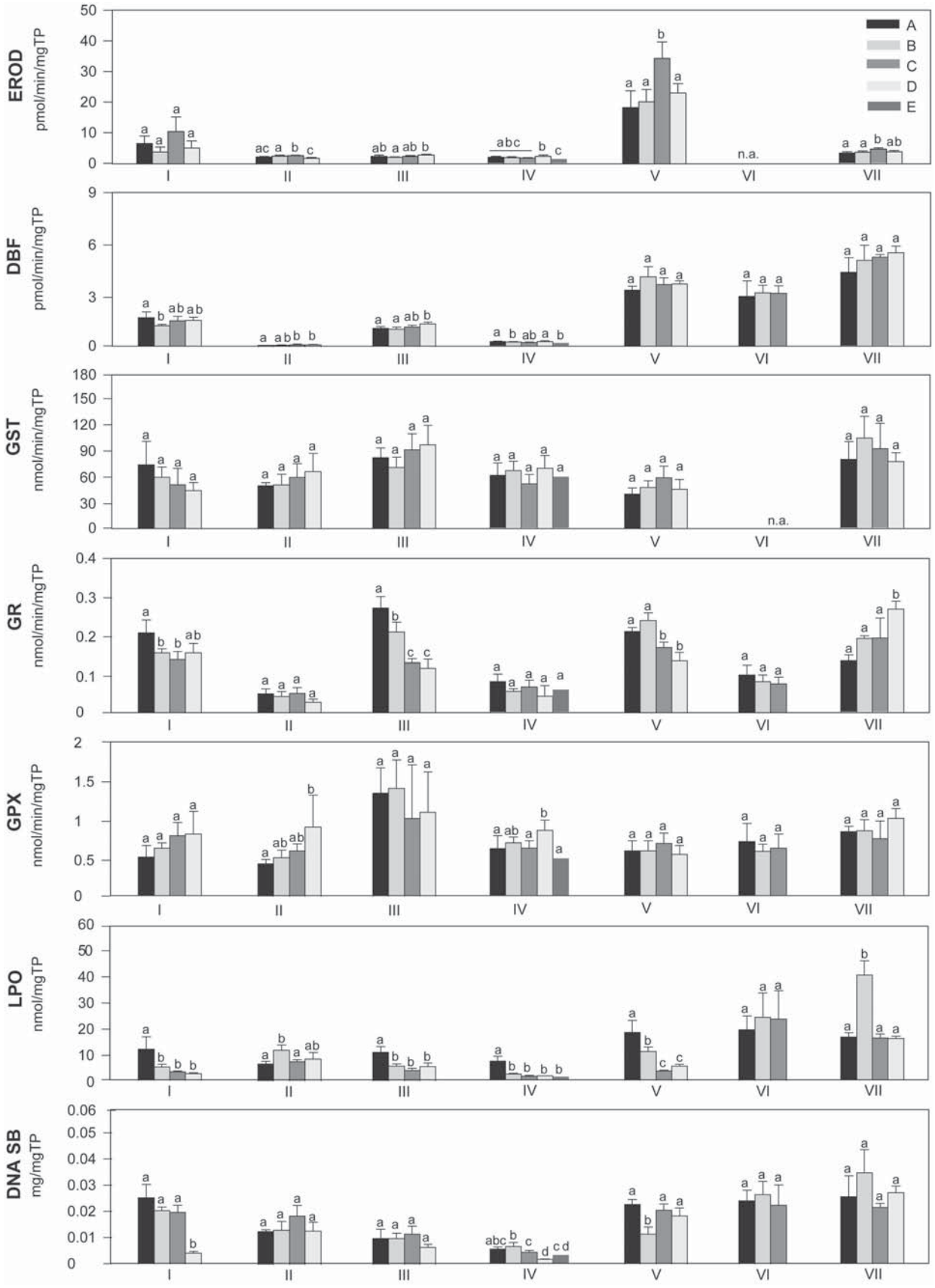


Figure 2. Biomarker results measured in hepatopancreas of specimens of *M. galloprovincialis* collected at approximately 0, 50, 100, 200, 400 and 1000 m from the outlet of seven LBMFFs, referred as I, II, III, IV, V, VI and VII. Letters a, b and c indicate groups statistically different ($p < 0.05$).

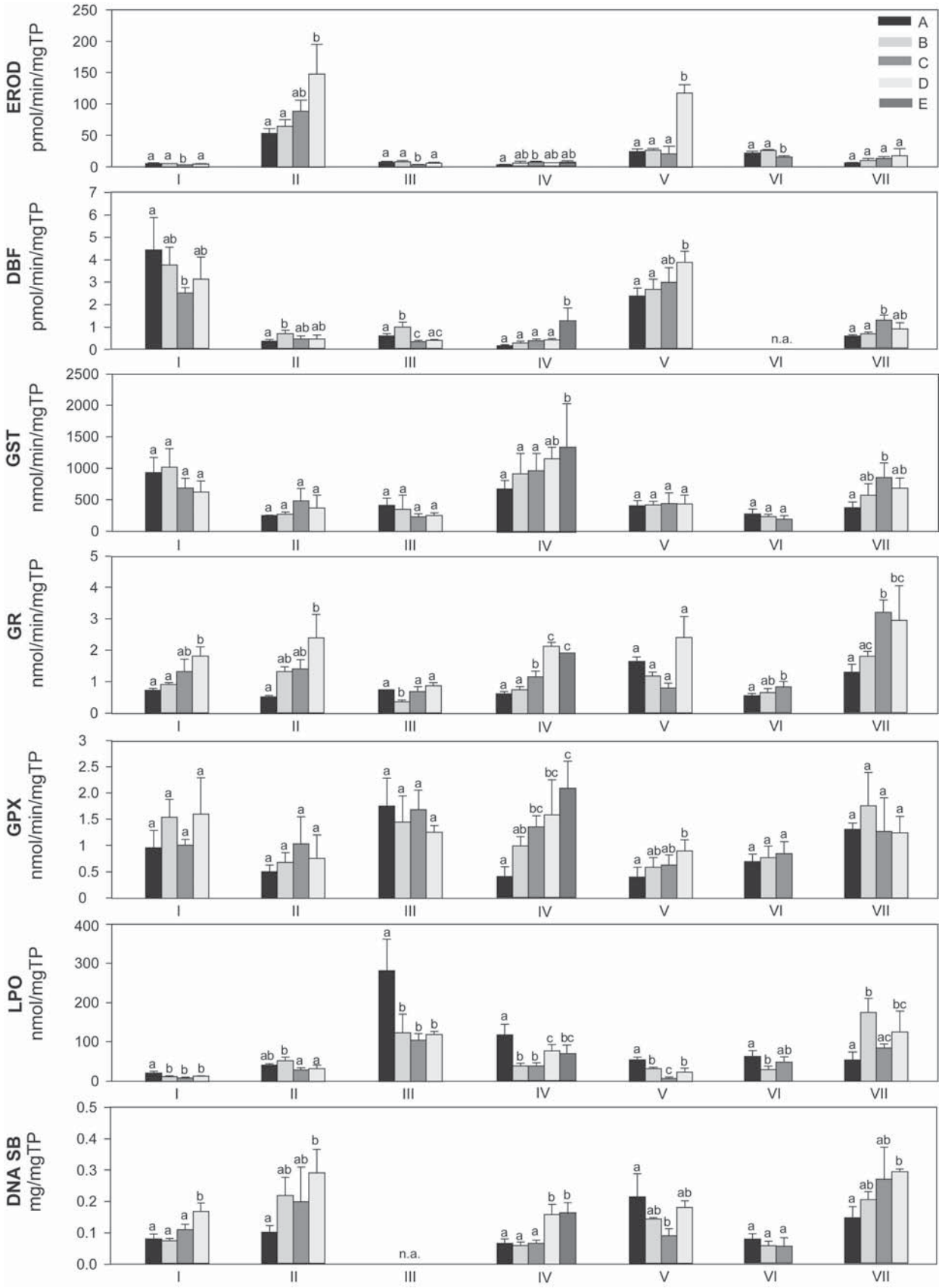


Figure 3. Biomarker results measured in gills of specimens of *M. galloprovincialis* collected at approximately 0, 50, 100, 200, 400 and 1000 m from the outlet of seven LBMFFs, referred as I, II, III, IV, V, VI and VII. Letters a, b and c indicate groups statistically different ($p < 0.05$).

Table 1. Summarized methodologies, according for the analysis of total proteins (TP) and a battery of biochemical biomarkers comprising the activities of the biotransformation enzymes EROD and DBF (phase I), and GST (phase II), the activities of the antioxidant enzymes GR and GPX, and the oxidative effects LPO and DNA strand breaks (DNA SB).

Analysis	Sample fraction	Method	Reference
TP	Homogenate and S_{15}	Objective: Concentration of total proteins 30 min incubation with ¼ diluted reagent (Bio-Rad Laborato-ries GmbH Cat. No. 5000-0006) Spectrophotometry - SOFTmax PRO package	Bradford (1976)
EROD	S_{15}	Objective: Rate of resorufin production Substrate: 7-ethoxyresorufin Reaction promoter: 1 mM NADPH Fluorometry: 485 nm (excitation), 580 nm (emission); measured every 15 min during 60 min	Gagné and Blaise (1993)
DBF	S_{15}	Objective: Rate of fluorescein production Substrate: 10 µM dibenzylfluorescein Reaction promoter: 1 mM NADPH Fluorometry: 485 nm (excitation) and 535 nm (emission); measured every 15 min during 60 min	Gagné and Blaise (1993)
GST	S_{15}	Objective: Rate of conjugation of 1-chloro-2,4-dinitorbenzene (CDNB) with reduced glutathione (GSH) Substrates: 1 mM CDBN and 1 mM GSH Spectrophotometry: 340 nm; measured every 5 min during 30 min	McFarland et al. (1999)
GR	S_{15}	Objective: Rate of NADPH consumption during the reduction of oxidized glutathione (GSSG) to GSH Substates: 10 mM oxidized glutathione and 1 mM NADPH Spectrophotometry: 340 nm; measured every 2 min during 10 min, 30°C	McFarland et al. (1999)
GPX	S_{15}	Objective: Rate of NADPH consumption during the reduction of GSSG to GSH. Tandem reaction with GR Substrate 1: 1 mM cumene hydroperoxide Substrate 2: 3 mM GSH and 1 mM NADPH with 67 units of GR Spectrophotometry: 340 nm; measured every 10 s during 3 min, 30°C	McFarland et al. (1999)
LPO	Homogenate	Objective: Concentration of malondialdehyde (MDA) Substrate: 0.67% thiobarbituric acid Spectrophotometry: 540 nm	Wills (1987)
DNA SB	Homogenate	Objective: Concentration of protein-free and alkali-labile DNA strands Reagents: 2% SDS, 10 mM EDTA, 10 mM Tris-base, 40 mM NaOH, 0.12 M KCl. Fluorometry: 360 nm (excitation) and 450 nm (emission)	Olive, 1988

Table 2. Physicochemical characteristics of effluents sampled at the land-based marine fish farms (LBMFFs) under study. Physicochemical characterization included yearly production, effluent salinity, pH, dissolved oxygen (O_2), supended solids (SS), total organic carbon (TOC), phosphates (PO_4), nitrites (NO_2), nitrates (NO_3) and ammonia (NH_3) (Carballeira et al., 2012a).

LBMFF	Production (t year^{-1})	Salinity (gL^{-1})	pH	O_2 (mgL^{-1})	O_2 (%)	SS (mgL^{-1})	TOC (mgL^{-1})	PO_4 (mgL^{-1})	NO_2 (mgL^{-1})	NO_3 (mgL^{-1})	NH_3 (mgL^{-1})
I	2250	32.4	7.48	8.3	79.6	23	5.64	0.22	0.086	0.190	0.85
II	292	34.7	7.92	8.4	80.1	17	5.35	0.42	0.072	0.200	0.45
III	308	34.8	7.94	8.5	82.2	18	7.05	0.25	0.069	0.192	0.63
IV	1194	34.7	7.63	7.8	79.1	21	8.04	0.29	0.080	0.205	0.78
V	348	34.2	7.80	8.9	87.3	16	6.09	0.24	0.078	0.141	0.56
VI	44	34.5	7.64	8.0	79.5	18	2.36	0.21	0.075	0.536	0.57
VII	285	34.3	7.59	8.2	80.3	14	4.70	0.23	0.066	0.206	1.04

Table 3. Pearson coeficients defining the correlations between the biomarkers, in gills (GL) and hepatopancras (HEP), and the distance to the contamination point source at the eight LBMFFs.

Farms	I		II		III		IV		V		VI		VII	
	GL	HEP	GL	HEP	GL	HEP	GL	HEP	GL	HEP	GL	HEP	GL	HEP
EROD	-0.327	0.001	**0.855	-0.273	-0.324	**0.614	0.234	-0.357	**0.870	0.325	-0.521	-	*0.654	0.281
DBF	-0.442	0.002	-0.046	*.664	*-0.484	**0.703	**0.832	*-0.429	**0.764	0.105	-	0.128	0.497	*0.528
GST	-0.022	*-0.531	0.327	0.442	*-0.449	0.415	**0.786	-0.005	0.107	0.147	*-0.543	-	*0.479	-0.155
GR	**0.893	-0.504	**0.870	*-0.572	*0.567	**-0.865	**0.922	-0.230	-0.107	**-0.781	*0.743	-0.465	**0.697	**0.879
GPX	0.347	*0.533	0.261	**0.665	-0.391	-0.237	**0.766	-0.288	**0.708	-0.110	0.316	-0.212	-0.104	0.360
LPO	-0.440	**-0.708	-0.480	0.020	*-0.590	*-0.532	0.014	*-0.545	*-0.613	**-0.736	-0.340	0.231	0.293	-0.330
ADN	**0.880	**-0.669	**0.628	0.045	-	0.030	**0.795	*-0.518	-0.215	-0.056	-0.464	-0.017	**0.662	-0.095

* $p < 0.05$; ** $p < 0.01$.

Table 4. Summarized results of Pearson analysis between pairs of biomarkers, in gills and hepatopancreas, at the seven LBMFFs. Only significant correlations are indicated.

		I	II	III	IV	V	VI	VII
GILLS	EROD	**DBF **LPO	**GST **GPX	**DBF ***GST	*DBF **GST **GPX	**DBF **GPX	*ADN	**DBF
	DBF	**EROD **GST *GR *LPO *ADN	*LPO	**EROD **GST **GR	*EROD **GST **GR **GPX **ADN	**EROD **GST **GPX	-	**EROD **GST *GR
	GST	**DBF	**EROD **GPX	**EROD **DBF	**EROD **DBF **GR **GPX **ADN	**DBF	-	**DBF **GR *ADN
	GR	*DBF **ADN	**ADN	**DBF	**DBF **GST *GPX **ADN	**ADN	-	*DBF **GST **ADN
	GPX	-	**EROD **GST	*LPO	**EROD **DBF **GST *GR **ADN	**EROD **DBF	-	-
	LPO	**EROD *DBF	*DBF	*GPX	-	**ADN	*ADN	-
	ADN	*DBF **GR	**GR	-	**DBF **GST **GR **GPX	**GR **LPO	*EROD *LPO	*GST **GR
HEPATOPANCREAS	EROD	-	**ADN	**DBF	**DBF **GST **GPX	*GST	-	*DBF
	DBF	*GST *GR	*GR *ADN	**EROD **GST *GR	**EROD *GST **GPX	-	**GPX	*EROD
	GST	*DBF **GR *LPO	*GR **GPX	**DBF	**EROD *DBF *GR *GPX	*EROD *GPX *LPO	-	-
	GR	*DBF **GST **LPO	*DBF *GST *GPX	*DBF *LPO	*GST	*LPO	**ADN	-
	GPX	-	**GST *GR	-	**EROD **DBF *GST	*GST	**DBF	-
	LPO	*GST **GR	-	*GR	-	*GST *GR	*ADN	**ADN
	ADN	-	**EROD *DBF	-	-	-	**GR *LPO	**LPO

Black: positive correlations; Grey: negative correlations.
* $p < 0.05$; ** $p < 0.01$.

2.5. Statistical analysis

One way ANOVA (Tukey's test) was used (after confirming the normal distribution of data) to identify significant differences ($p < 0.05$) in the results for each site along the spatial gradients, in order to evaluate whether or not there was a gradient of impact. Tukey's test was also used to identify significant differences ($p < 0.05$) in the mean results for each biomarker between scenarios, thus to establish the most significant biomarker responses in each study area. Pearson's correlation analysis was conducted to check any significant ($p < 0.01$ and $p < 0.05$) relationships between biomarkers and the distance from the fish farm outputs, so that additional information about the possible gradient of impact could be provided, and also to explore the relationships between pairs of biomarkers at each study area. The analyses were carried out by use of the statistical package SPSS Statistics 17.0.

3. Results

3.1. Effluent characterization

The physico-chemical parameters of all effluents are shown in table 2. They were similar to the average values determined for the output water from 18 LBMFFs in Galicia (Carballeira et al., 2012a).

3.2. Spatial variability of biomarkers

The results of the biomarker analysis in hepatopancreas and gills of native *M. galloprovincialis* are shown in Figure 2 and Figure 3, respectively. In these figures, the results of Tukey's test, which exhibits significant ($p < 0.05$) differences amongst sites along the spatial gradient in each scenario, are also displayed. Significant ($p < 0.05$) relationships between biomarker responses and the distance from the fish farm outlets are summarized in Table 3.

Pearson's correlation analysis revealed that biomarkers were correlated ($p < 0.05$) with the distance from the fish farm discharge outlets, particularly in gill tissue, except for farms I and III, where the number of biomarkers that varied significantly ($p < 0.05$) with the distance was similar in both tissues. In mussels from farm VI, biomarkers barely varied ($p < 0.05$) with the distance from the outlet, in either gills or hepatopancreas.

Most of the significant ($p < 0.05$) relationships between biomarkers measured in mussel gills and the distance from the source of contamination were direct, i.e. biomarker responses increased with distance. However, most of the biomarkers measured in hepatopancreas were inversely correlated with the distance from the outlet, i.e. biomarker responses decreased with the distance. For farm III most of the biomarkers were inversely correlated with the distance from the outlet, regardless of the tissue considered. LPO levels were always inversely correlated with the distance from the outlet, irrespective of the tissue and the study area.

According to the results of the Tukey's test, the biomarker responses measured in both tissues did not show pronounced gradients with significant ($p < 0.05$) differences amongst sites, except on rare occasions (e.g. GR and GPX activities measured in gills of mussels along the sampling gradient from farms IV and VII, respectively; GR activity measured in hepatopancreas of mussels along the sampling gradient from farm III). This indicated that the correlations between biomarkers and distance from the source of contamination identified by Pearson's correlation analysis corresponded to slight, gradual changes in biomarker responses.

3.3. Relationships between pairs of biomarkers

Significant ($p < 0.05$) relationships between pairs of biomarkers, measured in gills and hepatopancreas, are shown in Table 4.

Relationships between pairs of biomarkers differed between tissues. More biomarker responses were correlated with each other ($p <0.05$) in gills than in hepatopancreas.

Biotransformation enzymes were found to be usually directly correlated. EROD and DBF activities were correlated with each other ($p < 0.05$) in mussel gills for five out of seven scenarios, and in hepatopancreas for three out of seven scenarios. GST activity was more often correlated ($p < 0.05$) with DBF activity than with EROD activity. EROD and GST activities were correlated in gill tissue, for 3 out of 7 study areas, and in hepatopancreas, for 2 out of 7 study areas. The DBF and GST activities were correlated in gill tissue for 5 out of 7 study areas and in hepatopancreas for 3 out of 7 scenarios.

The activity of phase I biotransformation enzymes, specially DBF activity, were often directly correlated ($p < 0.05$) with the activities of antioxidant enzymes and with the effect biomarkers LPO and DNA strand breaks, both in gills and in hepatopancreas. This type of association was highlighted in mussels at farm IV, where correlations with GR and GPX activities, as well as with DNA strand breaks were observed in gills and/or hepatopancreas. The same happened in mussels from farms I, II and VI, where correlations with LPO and/or DNA strand breaks were observed in gills.

In some scenarios, the activities of antioxidant enzymes and the effect biomarkers were correlated with each other ($p < 0.05$), without being related to the activities of phase I biotransformation enzymes. LPO was correlated with DNA strand breaks in mussels sampled along the gradients from farms V (gills), VI (both tissues) and VII (hepatopancreas). GST, GR and GPX activities were correlated between each other or with LPO and/or DNA strand breaks in mussels sampled along the gradients from farms I (hepatopancreas), II (both tissues), III (both tissues), V (both tissues) and VII (gills).

3.4. Significant biomarker responses in the study areas

Finally, each study area was characterized according to the average levels of the biomarkers measured in gills. Mussels from the scenario of farm I were characterized by significantly higher ($p < 0.05$) DBF, GST and GPX activities than in the mussels sampled at the other areas. Mussels sampled on the gradient from farm II were characterized by high ($p < 0.05$) EROD activity and high amount of DNA strand breaks. Mussels sampled in the scenario of farm III exhibited significantly higher ($p < 0.05$) LPO levels than those sampled at the other study areas. Mussels sampled along the gradients from farms IV and VII exhibited significantly higher ($p < 0.05$) GST and GPX activities, and furthermore, those sampled along the gradient from farm VII showed higher LPO levels and more DNA strand breaks than mussel from the other scenarios. Mussels sampled along the gradient from farm V were characterized by significantly higher ($p < 0.05$) EROD and DBF activities, and by more DNA strand breaks than in the mussels from the other farms. Only EROD activity was significantly higher ($p < 0.05$) in the mussels from farm VI than in the mussels from the other areas.

4. Discussion

4.1. Are biomarker results indicative of stress?

In order to evaluate whether the data obtained in the present study indicate environmental stress, they were compared with previously published data obtained by analysis of *M. galloprovincialis* on the Atlantic coast of the Iberian Peninsula. These comparisons enabled estimation of the normal range of variation of the analyzed biomarkers in this species, integrating seasonal and geographical variations and the influence of different types of pollution.

We did not find any information about the levels of EROD and DBF activity in this species or in others of the same genus. Moreover, we could not compare our results on DNA strand break with those reported for *M. galloprovincialis*, because of differences in the analytical methodologies. EROD, DBF and DNA strand breaks activities appear to have been more commonly measured in freshwater mussels such as *Elliptio complanata* (Martín-Díaz et al., 2009), *Dreissena polymorpha* (Binelli et al., 2006; Ricciardi et al., 2006) and *Perna perna* (Pereira et al., 2011). The EROD activities observed in diverse tissues (soft tissues, pools of digestive and gonad tissues) of these mussels were of the same order of magnitude as those obtained in the present study in gills and hepatopancreas, except for mussels from the sampling gradients starting at farms II and V, in which values were significantly higher.

The GST activity was similar to that reported by other authors for *M. galloprovincialis*, in both gills (Bebianno et al., 2007; Lima et al., 2007; Vidal-Liñán et al, 2010) and hepatopancreas (Lima et al., 2007), although the mean value of the biomarker in gills obtained in the present study was higher, which may indicate an exceptional response of GST, possibly because of particular environmental stress conditions.

The GR and GPX activities in gills were also similar to those reported in various studies (Fernández et al, 2010; Lima et al., 2007; Maria et al., 2009; Vidal-Liñán et al, 2010), although the values obtained in the present study were lower in all cases. On the other hand, GR and GPX activities in hepatopancreas, reported by other authors (Lima et al., 2007; Maria et al., 2009) were much higher than the values obtained in the present study, even when the control conditions were under consideration. This may indicate different degrees of inhibition

depending on the tissue, probably because of the oxidative load of the LBMFF effluents.

Finally, the LPO levels in both tissues were higher than those reported by different authors (Box et al., 2007; Fernández et al., 2010; Lima et al, 2007; Martín-Díaz et al., 2009; Sureda et al., 2011), especially in gills, in which differences of two orders of magnitude were observed. This may indicate severe pro-oxidant conditions in the study areas.

4.2. Biomarker sensitivity in mussel tissues

Gills and hepatopancreas were found to have similar sensitivity to fish farm discharges. However, biomarkers measured in gills exhibited more significant correlations with the distance from the discharge outlets and with each other than those analyzed in the hepatopancreas. The gills therefore appeared to comprise the most significant and coordinated defence responses of *M. galloprovincialis* at a biochemical level as regards contamination from the LBMFFs. Similar results have been obtained with biomarkers from *M. galloprovincialis (*Bebianno et al.; 2007; Regoli and Principato, 1995; Soldatov et al., 2007) Furthermore, these authors found that the enzymatic activities in gills were not affected by internal factors and concluded that the antioxidant system in this bivalve species is tissue specific and that the antioxidant responses in gills better reflected aquatic environmental conditions.

One plausible explanation for these results is that the gills of filter feeders are in direct contact with water and pollutants present in this medium, and are therefore constantly forced to counteract the harmful effects of these substances and neutralize the oxidative load.

4.3. Biomarkers relationship with the contamination gradient

The hydrodynamic conditions of the areas under study facilitate the rapid dilution of the effluent and the contaminant load, and apart from the fish farm effluents, no other sources of contamination were found, except at farm I, which is sheltered by maritime infrastructures and is located close to an aluminium factory and a port. For these reason, the most heavily impacted zone was expected to be that closest to the outlet, and this impact was expected to decrease with increasing distance from the source of

contamination. In fact, a contamination gradient was previously indicated in this area by the $\delta^{15}N$ signal determined in macroalgae, anemonae and mussels (Rey-Asensio et al., 2010) and histopathological damages determined in mussels and clams (Carballeira et al., 2011). In this work, the measured biomarkers responded to this gradient, although the responses differed depending on the tissue and, to a lesser extent, the study area.

Biomarkers measured in hepatopancreas showed fewer significant correlations with the distance from the source of contamination. These results indicated that the effects of contamination were less important in this tissue, so that environmental contamination from fish farming activities cannot be reliably indicated by the most biochemical parameters analyzed in this tissue.

All biomarkers in gills, except for LPO, were directly correlated with the distance from the source of contamination at most scenarios. This direct correlation could be explained because native mussels from the closest sites to the effluents may have developed mechanisms for adapting to chronic contamination (Regoli and Pincipato, 1995), so that enzymatic defences are blocked in response to permanent stress conditions. It is also possible that the chronic chemical input had led to defective induction of the defence biomarkers (Hansson et al., 2006). Both adaptation and impairment of the enzymatic defence assemblage may have favoured oxidative stress, leading to the observed induction of LPO. Stress conditions in the vicinity of the discharge outlets were demonstrated by Carballeira et al. (2011), who observed histological damages in native mussels at some of the studied farms. Therefore, the contaminant load from the fish farms may have caused deterioration in mussel health closer to the output, which was detected at the biochemical level, because of the strong induction of LPO and the impairment of xenobiotic metabolization and antioxidant capacities, and at histological level. Stress conditions appeared to decrease, as shown by a decrease in LPO, and enzymatic functions appeared to recover with increasing distance from the contamination source.

The results of DNA strand breaks were more difficult to explain. DNA damage was expected to present the same pattern as LPO, because both can be induced as a consequence of oxidative stress (Valavanidis et al., 2006), and moreover, DNA damage has been reported to be mediated by products of

LPO (Cheung et al., 2002). Nevertheless, DNA strand breaks were inversely related to LPO and exhibited significantly lower values than in other marine mussels (Pereira et al., 2011). This could be explained by the absence of genotoxic chemicals in the farm effluents. Although genotoxic xenobiotics such as polychlorinated biphenyls (Marabini et al., 2011), pesticides (Bony et al., 2008) and dioxins (Schwarz and Appel, 2005) have been found to be trace components of fish feed (Bell et al., 2005; Nardelli et al., 2004; Rodil et al., 2007), other contaminants may prevail in fish farm discharges. Another possible explanation is that DNA damage was avoided by ROS scavenging carried out by antioxidant defences other than those considered in this study, e.g. superoxide dismutase, peroxidases and non enzymatic antioxidants (Van der Oost et al., 2003) and/or effective DNA repair mechanisms (Slupphaug et al., 2003). Nonetheless, none of these hypotheses explained the slight increase in DNA at the sites furthest from the contamination source. DNA damage remained at extremely low values in comparison with that observed in *P. perna* (Pereira et al., 2011), regardless of the distance considered, so the observed enhancement may not be significant in terms of health of the organisms.

4.4. Relationship between biomarkers

The EROD activity was related to DBF activity in gills at most scenarios, indicating the possible exposure of mussels to CYP inducers. It is known that the amount or activity of CYP enzymes can be enhanced in the presence of compounds that are substrates of the reactions that they catalyze (Rewitz et al., 2006; Snyder, 2000; Van der Oost et al., 2003), hence changes in enzyme performance may indicate the presence of such chemicals in the environment. EROD activity has been widely shown to be a suitable biomarker of exposure to lipophilic organic contaminants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins, furans, pesticides and pharmaceuticals, in aquatic invertebrates (Binelli et al., 2006; Orrego et al., 2005). DBF belongs to the CYP3A4 family of enzymes (Gagné et al., 2007), which is known to be involved in most drug metabolism reactions in humans (Haddad et al., 2007). Aquatic invertebrates have also been shown to respond to pharmaceutical products through the induction of DBF activity (Aguirre-Martínez et al., 2010; Gagné et at., 2007; Martín-Díaz et al., 2009). As mentioned above, several

lipophilic compounds have been found to be trace components of fish feed, and may be released within the particulate waste. Furthermore, antibiotics are commonly used in aquaculture to prevent and treat fish diseases, and surplus antibiotics are therefore released to the environment. The presence of different antibiotics and pesticides was confirmed in the surroundings of the studied LBMFFs by bioaccumulation studies (Rey-Asensio et al.,2010). Hence, it is likely that mussels were exposed to a complex mixture of organic contaminants originated from the fish farms and that these chemicals were responsible for the response of phase I biotransformation enzymes. This type of contamination seemed to prevail in the discharges from farms I, II and V, as the highest mean EROD levels and/or DBF activities were observed in gills of mussels from the respective sampling gradients.

Phase I of the xenobiotic metabolism often functions as a xenobiotic activation process, which leads to more reactive, and consequently, more toxic molecules than the original compound (Gonzalez, 2005; Gonzalez and Kimura, 2001). This may explain the association between EROD and DBF activities and the activity of antioxidant enzymes, and in some cases with biomarkers of effect, as in gills of mussels from farms I, II and V. Despite the observed correlations, the antioxidant defence system was not significantly enhanced in these organisms, except in mussels from farm I. Considering the deterioration in the gills of mussels close to the contamination source, the apparent lack of response of the antioxidant enzymes may reflect depletion or impairment of the defence mechanisms. This depletion may possibly be due to metabolism-mediated xenobiotic activation and subsequent oxidative stress, and/or to oxidative stress directly caused by other prooxidant contaminants present in farm effluents. The decay in the antioxidant defence system has previously been reported to be a consequence of pharmaceutical (Li et al., 2009), pesticide (Ceyhun et al., 2010; Kavitha and Rao, 2008) and metal (Atli and Canli, 2010) exposure in aquatic organisms. Trace concentrations of metals have also been detected in fish feed (Ikem and Egilla, 2008) in organisms close to the studied LBMFFs (Rey-Asensio et al., 2010).

Activities of EROD and/or DBF were linked to the activity of the phase II enzyme GST in mussels at most scenarios, indicating possible combination of biotransformation phases I and II. GST activity was higher than observed in

previous studies, especially in mussels from farms I, IV and VII. Nevertheless, EROD and DBF activities were not significantly induced in mussels close to these farms, at least farms IV and VII. GST activity has been found to be enhanced by exposure to organic xenobiotics and it is known that some xenobiotics can undergo phase II reactions directly, bypassing phase I because they already have an electrophilic group (Blanchette et al., 2007; Livingstone, 1998), or because they have already react with free radicals under oxidative stress (Sherratt and Hayes, 2002). Furthermore, the high GST activity may also mean that this enzyme was involved in other functions apart from phase II biotransformation. GST is considered to be part of the antioxidant defence system (Bocchetti et al., 2008b; Lima et al., 2007; Vidal-Liñán et al., 2010) through different pathways (Blanchette et al., 2007; Ketterer et al., 1990; Moreira and Guilhermino, 2005, Prohaska, 1980; Sherratt and Hayes, 2001; Vidal-Liñán et al., 2010). In fact, GST was correlated with the antioxidant enzymes in gills of mussels from the sampling gradients for farms IV and VII.

GPX exhibited significant activity in both scenarios, together with DNA damage, which was also significantly higher in gills of mussels from the farm VII sampling gradient, than in mussels from the other study areas. These results suggest that effluent from these two farms may contain electrophilic contaminants, metallic and organic, which induced oxidative stress in mussels.

Ammonia, the main metabolic residue of fish, has been found to enhance oxidative stress in fish (Ching et al., 2009; Hegazi et al., 2010; Pan et al., 2011) and effluents from farms IV and VII presented the highest concentration of ammonia of all farms (Carballeira et al., 2012a). Antifouling products such as sodium hypochlorite and an alkyl amine surfactant have also shown to produce oxidative stress in *M. galloprovincialis* (López-Galindo et al., 2010).

4.5. Role of biological tools in environmental monitoring

Conventionally, chemical analysis is one of the first steps (or even the only one) in the process of environmental quality assessment and monitoring. Chemical monitoring normally focuses on the detection of priority compounds inventoried by responsible organisations (Roose et al., 2011). Then, results may be integrated with biological or ecosystem measures to evaluate contamination effects and establish possible causative

relationships. However, when information about chemicals is not available and contaminant determination is difficult due to a fast dilution or dispersion processes, biological monitoring used in the first place may be a more efficient method to detect environmental problems (Carballeira et al., 2012c). OSPAR (1997, 2008) proposes the use of biological tools (bioassays and biomarkers) as the initial phase to evaluate environmental quality, integrating what it calls *early warning programmes*. Biological tools are seen as precursors to trigger more thorough research on causative sources, chemical tracing and more contaminant-specific biological effects. Although biological monitoring can also be conducted independent of chemical surveillance (Roose et al. 2011), and especially in the studied areas where chemical analysis has been proved to be ineffective.

The high dilution and dispersion of aquaculture effluents together with the difficulties in obtaining reliable information about chemical use in these facilities made the biological effect monitoring an appropriate approach to evaluate the potential environmental impact of LBMFFs in Galicia (Carballeira et al., 2012c). Evidences of the vague evaluation of the environmental quality status that may result from chemical analysis in this area were presented as body burdens measures of metals, pesticides and antibiotics in different marine species (Rey-Asensio et al., 2010). Most of these substances were detected in very low concentrations and did not show any spatial trend that could allow assessing the influence and potential impact of the farms.

Low or undetectable concentrations of contaminants should not lead to underestimate their presence or their possible impact on marine biota and coastal ecosystems. Chemicals may have synergistic interactions, even at low concentrations, amongst each other and with naturally occurring compounds (Amorós et al., 2000; Sharma et al., 2010; Taylor et al., 2005; Wah Chu and Chow, 2002). Furthermore, synthetic organic compounds such as antibiotics and pesticides may undergo biotic and abiotic degradation (Kümmerer, 2009; Tiryaki and Temur, 2010), resulting on the production of equally or more toxic metabolites than the parent compound (Kümmerer, 2009; Amorós et al., 2000).

Conversely to chemical analysis, the biological tools, such as biomarkers of effect, and different types of bioassays, have proved that biological

monitoring is the best way to detect impacts from LBMFFs (Carballeira et al., 2011, 2012a, 2012b, 2012c, 2012d).

5. Conclusions

A battery of biomarkers, which comprised biotransformation and antioxidant enzyme activities and oxidative effect parameters, was successfully measured in tissues of the mussel *Mytilus galloprovincialis*.

Biomarkers activities were correlated with farm production rate. In this way, the biggest (I and IV) and smallest (VI) farms showed the highest and lowest biomarkers activities respectively. However, only one biomarker (LPO) showed a clear induction of its activity when approaching disharge point, which may suggest environmental deterioration. Therefore, LPO measured in the gills and digestive gland of *M. galloprovincialis* proved to be a useful indicator of the environmental quality of areas affected by LBMFF discharges.

Acknowledgements

The present study was partly financed by the Spanish Government's National Plan for Marine Culture (JACUMAR, 2008): "Selection of indicators, determination of reference values, design of programmes, protocols and measures for environmental studies in aquaculture (INDAQUA)". Carlos Carballeira is grateful for funding from the University of Cadiz Predoctoral Fellowship Programme (Spain).

References

Abrahamson, A., Andersson, C., Jönsson, M. E., Fogelberg, O., Örberg, J., Brunström, B., Brandt, I., 2007. Gill EROD in monitoring of CYP1A inducers in fish—A study in rainbow trout (*Oncorhynchus mykiss*) caged in Stockholm and Uppsala waters. Aquat. Toxicol. 85, 1-8.

Aguado-Giménez, F., Marín, A., Montoya, S., Marín-Guirao, L., Piedecausa, A., García-García, B., 2007. Comparison between some procedures for monitoring offshore cage culture in western Mediterranean Sea: Sampling methods and impact indicators in soft substrata. Aquaculture 271, 357-370.

Aguirre-Martínez, G. V., Salamanca, M. J., Del Valls, T. A., Martín-Díaz, M. L., 2010. Biological and biochemical responses of *Carcinus maenas*: A consequence of pharmaceutical exposure. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 157, S16-S17.

Amorós, I., Connon, R., Garelick, H., Alonso, J.L., Carrasco, J.M., 2000. An assessment of the toxicity of some pesticides and their metabolites affecting a natural aquatic environment using the MicrotoxTM system. Water Sci. Technol. 42, 19-24.

Atli, G., Canli, M., 2010. Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. Ecotoxicol. Environ. Saf. 73, 1884-1889.

Backhaus, T., Altenburger, R., Arrhenius, Å., Blanck, H., Faust, M., Finizio, A., Gramatica, P., Grote, M., Junghans, M., Meyer, W., Pavan, M., Porsbring, T., Scholze, M., Todeschini, R., Vighi, M., Walter, H., Horst Grimme, L., 2003. The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. Cont. Shelf. Res. 23, 1757-1769.

Bebianno, M. J., Lopes, B., Guerra, L., Hoarau, P., Ferreira, A. M., 2007. Glutathione S-tranferases and cytochrome P450 activities in *Mytilus galloprovincialis* from the South coast of Portugal: Effect of abiotic factors. Environ. Int. 33, 550-558.

Bell, J. G., McGhee, F., Dick, J. R., Tocher, D. R., 2005. Dioxin and dioxin-like polychlorinated biphenyls (PCBs) in Scottish farmed salmon (*Salmo salar*): effects of replacement of dietary marine fish oil with vegetable oils. Aquaculture. 243, 305-314.

Binelli, A., Ricciardi, F., Riva, C., Provini, A., 2006. New evidences for old biomarkers: Effects of several xenobiotics on EROD and AChE activities in Zebra mussel (*Dreissena polymorpha*). Chemosphere. 62, 510-519.

Blanchette, B., Feng, X., Singh, B., 2007. Marine Glutathione S-Transferases. Mar. Biotechnol. 9, 513-542.

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., Pellegrini, D., Regoli, F., 2008a. Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquat. Toxicol. 89, 257-266.

Bocchetti, R., Lamberti, C. V., Pisanelli, B., Razzetti, E. M., Maggi, C., Catalano, B., Sesta, G., Martuccio, G., Gabellini, M., Regoli, F., 2008b. Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, Tapes philippinarum, and mussels, *Mytilus galloprovincialis*, from Adriatic sea. Mar. Environ. Res. 66, 24-26.

Bony, S., Gillet, C., Bouchez, A., Margoum, C., Devaux, A., 2008. Genotoxic pressure of vineyard pesticides in fish: Field and mesocosm surveys. Aquat. Toxicol. 89, 197-203.

Borja, Á., Rodríguez, J.G., Black, K., Bodoy, A., Emblow, C., Fernandes, T.F., Forte, J., Karakassis, I., Muxika, I., Nickell, T.D., Papageorgiou, N., Pranovi, F., Sevastou, K., Tomassetti, P., Angel, D., 2009. Assessing the suitability of a range of benthic indices in the evaluation of environmental impact of fin and shellfish aquaculture located in sites across Europe. Aquaculture 293, 231-240.

Box, A., Sureda, A., Galgani, F., Pons, A., Deudero, S., 2007. Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 531-539.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295-311.

Carballeira, C., De Orte, M.R., Viana, I.G., Carballeira, A., 2012a. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. Ecotoxicol. Environ. Saf. 78, 148-161.

Carballeira, C., Espinosa, J., Carballeira, A., 2011. Linking δ15N and histopathological effects in molluscs exposed in situ to effluents from land-based marine fish farms. Mar. Pollut. Bull. 62, 2633-2641.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, L., DelValls, T.A., 2012b. Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents. Mar. Environ. Res. 77, 12-22.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, L., DelValls, T.A., Carballeira, A., 2012c. Designing an integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas. J. Environ. Monit. 14, 1305-1316.

Carballeira, C., Viana, I., Carballeira, A., 2012d. δ15N values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms. J. Appl. Phycol., 1-11.

Ceyhun, S. B., Sentürk, M., Ekinci, D., Erdogan, O., Çiltas, A., Kocaman, E. M., 2010. Deltamethrin attenuates antioxidant defense system and induces the expression of heat shock protein 70 in rainbow trout. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 215-223.

Cheung, C. C. C., Zheng, G. J., Lam, P. K. S., Richardson, B. J., 2002. Relationships between tissue concentrations of chlorinated hydrocarbons (polychlorinated biphenyls and chlorinated pesticides) and antioxidative responses of marine mussels, *Perna viridis*. Mar. Pollut. Bull. 45, 181-191.

Ching, B., Chew, S. F., Wong, W. P., Ip, Y. K., 2009. Environmental ammonia exposure induces oxidative stress in gills and brain of *Boleophthalmus boddarti* (mudskipper). Aquat. Toxicol. 95, 203-212.

Crane M., Burton G.A., Culp J.M., Greenberg M.S., Munkittrick K.R., Ribeiro, R., Salazar, M.H., St-Jean, S.D., 2007. Review of aquatic in situ approaches for stressor and effect diagnosis. Integr. Environ. Assess. Manage. 3, 234–245.

Duke, T.W., Mount, D.I., 1991. Toxic Effects on Individuals, Populations and Aquatic Ecosystems and Indicators of Exposures to Chemicals, in: B.D.G. Robert G. Tardiff (Ed.), Methods for Assessing Exposure of Human and Non-Human Biota. John Wiley & Sons Ltd, University of California, 417.

Fernández, B., Albentosa, M., Viñas, L., Franco, A., González, J., Campillo, J., 2010. Integrated assessment of water quality of the Costa da Morte (Galicia, NW Spain) by means of mussel chemical, biochemical and physiological parameters. Ecotoxicology. 19, 735-750.

Gagné, F., André, C., Cejka, P., Gagnon, C., Blaise, C., 2007. Toxicological effects of primary-treated urban wastewaters, before and after ozone treatment, on freshwater mussels (*Elliptio complanata*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 145, 542-552.

Gagné, F., Blaise, C., 1993. Hepatic metallothionein level and mixed function oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. Water Res. 27, 1669-1682.

Gonzalez, F. J., 2005. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. Mutat. Res. 569, 101-110.

Gonzalez, F. J., Kimura, S., 2001. Understanding the role of xenobiotic-metabolism in chemical carcinogenesis using gene knockout mice. Mutat. Res. 477, 79-87.

Haddad, A., Davis, M., Lagman, R., 2007. The pharmacological importance of cytochrome CYP3A4 in the palliation of symptoms: review and

recommendations for avoiding adverse drug interactions. Support Care Cancer 15, 251-257.

Handy, R.D., Galloway, T.S., Depledge, M.H., 2003. A Proposal for the Use of Biomarkers for the Assessment of Chronic Pollution and in Regulatory Toxicology. Ecotoxicology 12, 331-343.

Hansson, T., Schiedek, D., Lehtonen, K. K., Vuorinen, P. J., Liewenborg, B., Noaksson, E., Tjärnlund, U., Hanson, M., Balk, L., 2006. Biochemical biomarkers in adult female perch (*Perca fluviatilis*) in a chronically polluted gradient in the Stockholm recipient (Sweden). Mar. Pollut. Bull. 53, 451-468.

Hegazi, M. M., Attia, Z. I., Ashour, O. A., 2010. Oxidative stress and antioxidant enzymes in liver and white muscle of *Nile tilapia* juveniles in chronic ammonia exposure. Aquat. Toxicol. 99, 118-125.

Ikem, A., Egilla, J., 2008. Trace element content of fish feed and bluegill sunfish (*Lepomis macrochirus*) from aquaculture and wild source in Missouri. Food Chem. 110, 301-309.

Kavitha, P., Rao, J. V., 2008. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, *Gambusia affinis*. Environ. Toxicol. Pharmacol. 26, 192-198.

Ketterer, B., Meyer, D. J., Taylor, J. B., Pemble, S., Coles, B., Fraser, G., 1990. GST and protection against oxidative stress. Taylor and Francis, Bristol.

Kullman, M.A., Podemski, C.L., Kidd, K.A., 2007. A sediment bioassay to assess the effects of aquaculture waste on growth, reproduction, and survival of *Sphaerium simile* (Say) (Bivalvia: Sphaeriidae). Aquaculture 266, 144-152.

Kümmerer, K., 2009. Antibiotics in the aquatic environment – A review – Part II. Chemosphere 75, 435-441.

Li, Z.-H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Randak, T., 2009. Responses of antioxidant status and Na+-K+-ATPase activity in gill of rainbow trout, *Oncorhynchus mykiss*, chronically treated with carbamazepine. Chemosphere. 77, 1476-1481.

Lima, I., Moreira, S. M., Osten, J. R.-V., Soares, A. M. V. M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere. 66, 1230-1242.

Livingstone, D. R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. . Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 120, 43-49.

López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J. F., Rubio, D., Mancera, J. M., Solé, M., 2010. Sublethal responses of the common mussel (*Mytilus galloprovincialis*) exposed to sodium hypochlorite and Mexel®432 used as antifoulants. Ecotoxicol. Environ. Saf. 73, 825-834.

Marabini, L., Calò, R., Fucile, S., 2011. Genotoxic effects of polychlorinated biphenyls (PCB 153, 138, 101, 118) in a fish cell line (RTG-2). Toxicol. In Vitro. 25, 1045-1052.

Maria, V., Santos, M., Bebianno, M., 2009. Biomarkers of damage and protection in *Mytilus galloprovincialis* cross transplanted in Ria Formosa Lagoon (Portugal). Ecotoxicology 18, 1018-1028.

Martín-Díaz, M. L., Blasco, J., Sales, D., DelValls, T. A., 2008. Field validation of a battery of biomarkers to assess sediment quality in Spanish ports. Environ. Pollut. 151, 631-640.

Martín-Díaz, M. L., Gagné, F., Blaise, C., 2009. The use of biochemical responses to assess ecotoxicological effects of Pharmaceutical and Personal Care Products (PPCPs) after injection in the mussel *Elliptio complanata*. Environ. Toxicol. Pharmacol. 28, 237-242.

McFarland, V.A., Inouye, L.S., Lutz, C.H., Jarvis, A.S., Clarke, J.U., McCant, D.D., 1999. Biomarkers of Oxidative Stress and Genotoxicity in Livers of Field-Collected Brown Bullhead, *Ameiurus nebulosus*. Arch. Environ. Contam. Toxicol. 37, 236-241.

Moreira, S. M., Guilhermino, L., 2005. The Use of *Mytilus Galloprovincialis* Acetylcholinesterase and Glutathione S-Transferases Activities as Biomarkers of Environmental Contamination Along the Northwest Portuguese Coast. Environ. Monit. Assess. 105, 309-325.

Nardelli, V., Palermo, C., Centonze, D., 2004. Rapid multiresidue extraction method of organochlorinated pesticides from fish feed. J. Chromatogr. 1034, 33-40.

Olive, P.L., 1988. DNA precipitation assay: A rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11, 487-495.

Orrego, R., Jiménez, B., Bordajandi, L. R., Gavilán, J. F., Inzunza, B., Abad, E., González, M. J., Rivera, J., Barra, R., 2005. EROD induction and PCDD/F levels in fish liver from the Biobio River in Chile. Chemosphere. 60, 829-835.

OSPAR, C., 2003. The OSPAR Integrated Report 2003 on the Eutrophication Status of the OSPAR Maritime Area based upon the first application of the Comprehensive Procedure, Eutrophication series. OSPAR, Paris.

OSPAR, C., 2008. Background document on biological effects monitoring techniques, Assessment and Monitoring. OSPAR Commission, London, 122.

Pan, C. H., Chien, Y. H., Wang, Y. J., 2011. Antioxidant defence to ammonia stress of characins (*Hyphessobrycon eques Steindachner*) fed diets supplemented with carotenoids. Aquacult. Nutr. 17, 258-266.

Pandard, P., Devillers, J., Charissou, A.M., Poulsen, V., Jourdain, M.J., Ferard, J.F., Grand, C., Bispo, A., 2006. Selecting a battery of bioassays for ecotoxicological characterization of wastes. Sci. Total Environ. 363, 114-125.

Pereira, C. D. S., Martin-Díaz, M. L., Zanette, J., Cesar, A., Choueri, R. B., Abessa, D. M. d. S., Catharino, M. G. M., Vasconcellos, M. B. A., Bainy, A. C. D., de Sousa, E. C. P. M., Del Valls, T. A., 2011. Integrated biomarker responses as environmental status descriptors of a coastal zone (São Paulo, Brazil). Ecotoxicol. Environ. Saf. 74, 1257-1264.

Peters, C., Ahlf, W., 2005. Reproduction of the estuarine and marine amphipod *Corophium volutator* (Pallas) in laboratory for toxicity testing. Chemosphere 59, 525-536.

Peters, L. D., Livingstone, D. R., 2001. Induction of molluscan cytochrome P450 monooxygenase system as a biomarker of organic pollution in environmental monitoring In: Ph. Garrigues, H. B., C.H. Walker, J.-F. Narbonne, (Ed.), Biomarkers in Marine Organisms: A Practical Approach. Elsevier Science, Amsterdam, pp. 572.

Prohaska, J. R., 1980. The glutathione peroxidase activity of glutathione S-transferases. Biochimica et Biophysica Acta (BBA) - Enzymology. 611, 87-98.

Ramos-Gómez, J., Coz, A., Viguri, J. R., Luque, A., Martín-Díaz, M. L., Delvalls, T. A., 2011. Biomarker responsiveness in different tissues of caged *Ruditapes philippinarum* and its use within an integrated sediment quality assessment. Environ. Pollut. 159, 1914-1922.

Read, P., Fernandes, T., 2003. Management of environmental impacts of marine aquaculture in Europe. Aquaculture 226, 139-163.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat. Toxicol. 31, 143-164.

Rewitz, K. F., Styrishave, B., Løbner-Olesen, A., Andersen, O., 2006. Marine invertebrate cytochrome P450: Emerging insights from vertebrate and insect analogies. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143, 363-381.

Rey-Asensio, A., Carballeira, C., Viana, I. G., Carballeira, A., Biomonitorización de los efluentes de piscifactorías marinas instaladas en tierra: Bioacumulación de microcontaminantes, 2010. In: Rey-Méndez M., L. C., Fernández Casal J., Guerra A., (Ed.), Foro dos Recursos mariños e da Acuicultura das Rías galegas XIII, Vol. 13. USC, O Grove, pp. 201-218.

Ricciardi, F., Binelli, A., Provini, A., 2006. Use of two biomarkers (CYP450 and acetylcholinesterase) in zebra mussel for the biomonitoring of Lake Maggiore (northern Italy). Ecotoxicol. Environ. Saf. 63, 406-412.

Rodil, R., Carro, A. M., Lorenzo, R. A., Cela, R., 2007. Multicriteria optimisation of a simultaneous supercritical fluid extraction and clean-up procedure for the determination of persistent organohalogenated pollutants in aquaculture samples. Chemosphere. 67, 1453-1462.

Rodríguez-Gallego, L., Meerhoff, E., Poersch, L., Aubriot, L., Fagetti, C., Vitancurt, J., Conde, D., 2008. Establishing limits to aquaculture in a protected coastal lagoon: Impact of *Farfantepenaeus paulensis* pens on water quality, sediment and benthic biota. Aquaculture 277, 30-38.

Roose, P., Albaigés, J., Bebianno, M.J., Camphuysen, C., Cronin, M., de Leeuw, J., Gabrielsen, G., Hutchinson, T., Hylland, K., Jansson, B., Jenssen, B.M., Schulz-Bull, D., Szefer, P., Webster, L., Bakke, T., Janssen, C., 2011. Monitoring Chemical Pollution in Europe's Seas: Programmes, Practices and Priorities for Research, Marine Board Position Paper. Marine Institute, Ostend, Belgium, 108.

Sanchez, W., Porcher, J.-M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. TrAC, Trends Anal. Chem. 28, 150-158.

Schwarz, M., Appel, K. E., 2005. Carcinogenic risks of dioxin: Mechanistic considerations. Regul. Toxicol. Pharmacol. 43, 19-34.

Sharma, T., Ettensohn, C.A., 2010. Activation of the skeletogenic gene regulatory network in the early sea urchin embryo. Development 137, 1149-1157.

Sherratt, P. J., Hayes, J. D., 2002. Glutathione S-transferases. Enzyme Systems that Metabolise Drugs and Other Xenobiotics. John Wiley & Sons, Ltd, pp. 319-352.

Slupphaug, G., Kavli, B., Krokan, H. E., 2003. The interacting pathways for prevention and repair of oxidative DNA damage. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 531, 231-251.

Snyder, M. J., 2000. Cytochrome P450 enzymes in aquatic invertebrates: recent advances and future directions. Aquat. Toxicol. 48, 529-547.

Soldatov, A., Gostyukhina, O., Golovina, I., 2007. Antioxidant enzyme complex of tissues of the bivalve *Mytilus galloprovincialis* Lam. under normal and oxidative-stress conditions: A review. Appl. Biochem. Microbiol. 43, 556-562.

Taylor, R.L., Caldwell, G.S., Bentley, M.G., 2005. Toxicity of algal-derived aldehydes to two invertebrate species: Do heavy metal pollutants have a synergistic effect? Aquat. Toxicol. 74, 20-31.

Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A. Toxicol. In Vitro 22, 1128-1135.

Tiryaki, O., Temur, C., 2010. The Fate of Pesticide in the Environment. J. Biol. Environ. Sci. 4, 29-38.

Tovar, A., Moreno, C., Mánuel-Vez, M.P., García-Vargas, M., 2000. Environmental impacts of intensive aquaculture in marine waters. Water Res. 34, 334-342.

Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: Cytotoxicity and interactions with cytochrome P450 1A. Toxicol. In Vitro. 22, 1128-1135.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178-189.

Van der Oost, R., Beyer, J., Vermeulen, N. P. E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Vidal-Liñán, L., Bellas, J., Campillo, J. A., Beiras, R., 2010. Integrated use of antioxidant enzymes in mussels, *Mytilus galloprovincialis*, for monitoring pollution in highly productive coastal areas of Galicia (NW Spain). Chemosphere. 78, 265-272.

Wah Chu, K., and Chow, K. L., 2002. Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquat. Toxicol. 61, 53-64.

Wills, E.D., 1987. Evaluation of lipid peroxidation in lipids and biological membranes, in: K. Snell, Mullack, B. (Ed.), Biochemical Toxicology: A Practical Approach. Press Oxford, Oxford, 127-152.

Wright, D.A., Welbourn, P., 2002. Environmental Toxicology. Cambridge University Press, Cambridge University.

Zitko, V., 2001. Analytical chemistry in monitoring the effects of aquaculture: one laboratory's perspective. ICES J. Mar. Sci. 58, 486-491.

Las 35 palabras más usadas en esta tesis